Fall 1975

CHEMICAL STUDIES ON THE TOXINS OF THE MARINE DINOFLAGELLATE GONYAULAX TAMARENSIS AND THEIR ANALYSIS BY THIN-LAYER CHROMATOGRAPHY - FLUOROMETRY

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by

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A THESIS

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Doctor of Philosophy

Graduate School

Department of Biochemistry

September, 1975

This thesis has been examined and approved.

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July 25, 1975

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ACKNOWLEDGEMENT

The author wishes to express his gratitude to Dr. Miyoshi Ikawa for his interest, counsel and guidance. His insight and expertise have been invaluable throughout this study. Sincere thanks also go to Dr. James Wolfram and John Wakefield for their help with the fluorometry. The help and suggestions of my wife, Betty, along with her constant encouragement is acknowledged with thanks.

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CHEMICAL STUDIES ON THE TOXINS OF THE MARINE DINOFLAGELLATE

GONYAULAX TAMARENSIS AND THEIR ANALYSIS BY

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Recent outbreaks of paralytic shellfish poisoning (PSP) in previously unaffected coastal areas of New England have generated renewed interest in the phenomenon commonly referred to as a "red tide". At the initiation of this work, none of the toxins produced by Gonyaulax tamarensis had been isolated or identified. The major objectives of this study were to isolate and characterize the poisons from clams (Mya arenaria) and to develop a rapid method for determining the level of poison present in shellfish. Also, a simple method for evaluating fractions obtained during the isolation of toxic components was sought.

Three toxins have been purified from soft shell clams (Mya arenaria) which had become highly toxic during the Gonyaulax tamarensis red tide which affected the central New England coast in September 1972. The toxins were extracted from the clams with dilute acidic ethanol and isolated by chromatography on weak acid cation exchange resin columns and on gel filtration resins. A minor toxin, purified to a potency of 2,800 Mouse Units (MU) per mg was shown to be identical to saxitoxin (STX) in its thin-layer chromatographic behavior, its color reactions with various chromatographic spray reagents, and its effects on mice. Two very similar toxins, major toxin H and

major toxin L, were purified to a potency of 1,800 and 4,200 MU per mg respectively. Both of these toxins behaved differently from saxitoxin on weak cation exchange resin columns, thin-layer chromatography, and in their color reactions with various spray reagents. The effects of major toxins H and L on mice were indistinguishable from either saxitoxin or the minor toxin.

All of the G. tamarensis poisons were white hygroscopic, amorphous solids; soluble in water and lower alcohols and insoluble in lipid solvents. Like saxitoxin, none of the G. tamarensis toxins showed any visible or ultra-violet absorption above 220 nm.

A new in situ thin-layer chromatographic (TLC)-fluorometric method for the detection and quantitation of the paralytic shellfish poisons has been developed. This assay involves separation of the poisons by TLC, followed by reaction of the poisons on the TLC plate with 1% hydrogen peroxide at 100°C. As little as 40 ng (0.2 MU, 0.1 nM) of saxitoxin can be quantitated. This assay provides a means of distinguishing between major toxin H, major toxin L and saxitoxin (minor toxin), quantitating each toxin individually in a mixture and estimating their combined potency. The in situ TLC-fluorometric assay also suggested that the less basic toxins present in clams exposed to G. tamarensis may breakdown to give saxitoxin and that major toxin L can be converted to major toxin H, thus indicating a similarity in chemical structure between the three toxins.

INTRODUCTION

Dinoflagellates make up an important part of the marine plankton. In most areas of the ocean they are second only to the diatoms in abundance and as primary producers of organic matter. In tropical and subtropical waters dinoflagellates normally outnumber the diatoms (1). Dinoflagellates are found in fresh, brackish and salt water. They are single celled organisms ranging in size from 7 μ to 2 mm usually equipped with two flagella. A few species form linear colonies consisting of a few cells joined in tandem. Both naked and armored species exist. Armored dinoflagellates are covered with hard plates of polysaccharide material. The nutrition of dinoflagellates is varied. Some contain photosynthetic pigments and feed holophytically, fixing carbon dioxide in the water in the presence of sunlight. Others feed holozoically, consuming other organisms such as diatoms, flagellates and other dinoflagellates (2). Even the photosynthetic dinoflagellates may require certain complex organic compounds, such as, thiamine, biotin, Vitamin B_{12} (3) and humic and fulvic acids (4) for growth and reproduction. Wood (1) made the generalization that "On the whole, the oceanic and planktonic dinoflagellates tend to be holozoic, and the neritic forms holophytic or facultatively so...". Because they possess attributes of both plants and animals, the dinoflagellates have been classified at different times as Protozoa, algae and Protista. Halstead (5) suggests the term "plant-animals" used by Hunter.

The abundance and seasonal distribution of the dinoflagellates, like the diatoms, are effected by an annual cycle of events related to

temperature, salinity, light, nutrient and current regimes (4). Occasionally, for reasons not fully understood, the rate of reproduction of a particular dinoflagellate may increase rapidly and the organism may reach concentrations as high as 50,000 cells per ml (3). These spectacular outbursts or blooms of phytoplankton production are usually monogeneric or monospecific (1). At concentrations above 20,000 cells per ml, the water may appear red, yellow, orange or green depending upon the species responsible. This condition, regardless of color, is often referred to as a "red tide". Other organisms associated with "red tides" are diatoms and smaller flagellates. A bloom usually reaches its peak within 2 to 3 weeks and vanishes within another week or two. In adverse environmental conditions some dinoflagellates encyst and settle out of the water column. These dormant forms may serve as seed populations for future blooms. A number of marine dinoflagellates have been found to produce toxic metabolites. Blooms of certain of these organisms have been associated with the mortality of fish and other animals, e.g., Gymnodinium breve in the Gulf of Mexico (6).

Paralytic Shellfish Poisoning (PSP)

PSP is a severe form of food intoxication which occurs in widely scattered areas of the world (5). Symptoms appear within an hour after consumption of contaminated shellfish. Preliminary indications of poisoning, such as a tingling sensation in the lips and tongue, may occur within a few minutes, followed by a feeling of numbness in the legs, arms and neck. In severe cases, failure of muscle coordination is usually accompanied by a feeling of lightness. Prominent symptoms are loss of voice, incoherence of speech and constrictive sensations in the throat. Other symptoms which may also be

present are weakness, dizziness, muscular pain, prostration, headache, salivation, rapid pulse (80-100 per min.), intense thirst, difficulty in swallowing, perspiration, anuria and a vague feeling of discomfort. Gastrointestinal symptoms including nausea, vomiting, diarrhea and abdominal pain are less common (5) except in Atlantic Coast cases (7). Patients generally remain conscious. Muscular twitching and convulsions are rare. In fatal cases, death generally occurs within a period of 2 to 12 hours as a result of respiratory paralysis. If a person survives for 24 hours, he will usually recover showing no lasting effects. There is no specific antidote for PSP. Drug therapy has been used with varying degrees of success. Artificial respiration has been the most successful treatment.

Toxic shellfish are found sporadically in many areas of the world. The problem is more or less persistent in certain areas, including the Pacific coast of North America, the Bay of Fundy, the North Sea, the English Channel and portions of South Africa (8). The sporadic occurrence of PSP led to much early speculation about the origin of the poison. Copper salts present in sea water, putrification or diseases of the shellfish and contaminated water were some of the possibilities proposed (5). The causative agent for mussel poisoning along the Pacific coast of North America was finally demonstrated in 1937 by Sommer and Meyer and co-workers at the University of California (9, 10). These workers found: 1) there was a close correlation between the concentration of Gonyaulax catenella in the water around the mussel beds and the toxic levels of shellfish; 2) non-toxic mussels became poisonous when placed in water containing G. catenella; and 3) aqueous extracts of this plankton organism produced effects

similar to PSP in mice. Following the initial demonstration of the relationship between G. catenella, an armored dinoflagellate, and PSP in California, several other dinoflagellates were implicated in outbreaks of PSP in other areas of the world. These included Pyrodinium phoneus along the Belgium Coast, Gonyaulax tamarensis in the North Sea and along the northeast Atlantic coasts of North America and England, Gonyaulax acatenella along the coast of British Columbia.

The rate of toxin accumulation or loss by shellfish depends upon the concentration of the toxic dinoflagellate, the bivalve species exposed and its efficiency in filtering. The anatomical distribution of poison in shellfish is not uniform. The poison is generally found concentrated in different organs depending on the species and time of year. Mussels (Mytilus californianus) accumulate the poison more quickly than other shellfish and have been observed to become too toxic for human consumption when as few as 200 G. catenella cells were found per ml of water (11). Mussels appear to have a mechanism for binding the poison in the dark gland (hepatopancreas) and gradually destroying or excreting the bound poison so that, shortly after the bloom subsides, they are safe again for human consumption. Soft-shell clams (Mya arenaria) concentrate the poison quite rapidly when exposed to G. tamarensis. In summer, when the soft-shelled clams are exposed to the toxic dinoflagellate, the majority of the poison is found in the digestive gland and lesser amounts in the gills and gonads. In autumn, the gills contain about the same level of poison as in the summer, but the majority of poison is gone from the digestive gland (4). In contrast to many bivalves that release PSP within a few weeks following a toxic dinoflagellate bloom, Alaska butter clams

(Saxidomus giganteus) may retain the poison for at least two years (12, 3, 13). This property of butter clams made them a readily available and therefore important source of poison for studies on PSP. The poison apparently moves from the hepatopancreas to the siphon, where it is stored. The distribution of poison in the siphon appears to correspond to the areas of melanin pigmentation. Price and Lee (13, 14, 15) demonstrated an interaction between PSP and natural and synthetic melanin in vitro which was reversible and electrostatic in nature, closely resembling the interaction between PSP and weak acid cation exchange resins. Melanin, a metabolite of tyrosine, contains free carboxyl and phenolic hydroxyl groups which could function as cation exchangers. The observed binding of PSP to melanin was greatly influenced by pH and cation concentration. These authors hypothesized that the melanin in butter clam siphons may function as a protective mechanism for the clam.

A correlation has been observed between the resistance of a particular species of shellfish to saxitoxin and its ability to accumulate the poison. For example, the oyster (Crassostrea virginica), which is relatively sensitive to saxitoxin, is able to accumulate only very low levels of poison, while the mussel (Mytilus edulis) which is resistant to saxitoxin concentrates high levels of poison (16).

Detection and Quantitation of PSP

Bivalves from areas of the world affected by outbreaks of PSP must be continually monitored for the presence of poison. Since many edible species are relatively unaffected by the poison , there is no visible difference between safe and highly poisonous organisms.

Three general types of assays have been used for this purpose: 1) serological assays, 2) chemical assays and 3) biological assays. Of the three, only the mouse bioassay has gained wide use.

Johnson et al. (17) described the preparation of PSP-protein conjugates with haptenic properties by formaldehyde condensation, and production of antisera to PSP in rabbits. The PSP-protein conjugate was absorbed to either tanned sheep blood cells for use in a haemagglutination inhibition test for PSP or to bentonite particles for use in a bentonite flocculation inhibition test for PSP (18). The haemagglutination inhibition test for PSP was considerably more sensitive than the mouse test but the blood cell preparation was very unstable. The bentonite preparation had a considerably longer shelf life but the bentonite flocculation inhibition test for PSP was only about as sensitive as the mouse test. Although both of these serological tests are very specific for PSP, they have not been widely used since reagent preparation and test procedures are complex and time-consuming.

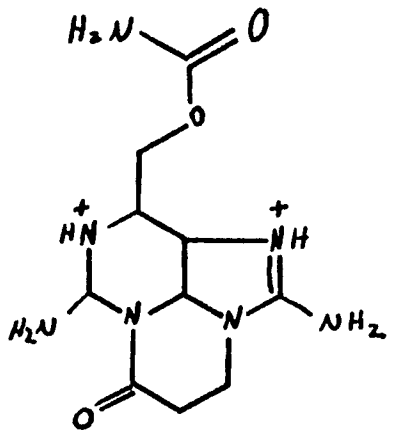
McFarren et al. (19) described a colorimetric test for the determination of PSP. The test involved extraction of the poison from shellfish meat with trichloroacetic acid, selective adsorption and elution of the poison on a cation exchange resin (Amberlite XE-64) and reaction of the eluate with picric acid (Jaffe Test). Later an additional step was added involving extraction of the unreacted picric acid after color development with 25% pyridine in ethyl acetate (20). The column did not completely remove substances which interfere with the Jaffe Test, and the age and state of decomposition of the shellfish affected test results. This modified Jaffe Test is not in wide use since it is complex, time-consuming and less sensitive than the mouse test.

Neve (21) reported a chemical assay involving the coupling of saxitoxin with 1-fluoro-2,4-dinitrobenzene. This procedure is relatively nonspecific and less sensitive than the mouse test.

Bates and Rapoport (22) recently described a chemical assay for saxitoxin involving alkaline hydrogen peroxide oxidation of the poison to 8-amino-6-hydroxymethyl-2-iminopurine-3(2H)-propionic acid (Fig. 1, structure 4) and determination of its fluorescence in solution at pH 5. Saxitoxin was extracted from ground shellfish meat with 0.5M trichloroacetic acid and adsorbed onto a weak acid cation exchange resin (Bio-Rex 70). The column was then eluted with pH 5 acetate buffer, water and finally 0.5M HCl. The 0.5M HCl eluate which contains the poison was divided into two portions. One portion was reacted with H_2O_2 at 20°C; the other was used as a blank. This chemical assay is capable of determining as little as .003 ug of saxitoxin per g of shellfish.

Bioassays for PSP using a wide range of test organisms, including rabbits, frogs, mice, rats, kittens, Guinea pigs, dogs, fish and sea urchins, have been reported (5). Of these, only the mouse test, first employed by Sommer and Meyer in 1937, is in general use. The mouse test involves extraction of the poison from shellfish and intraperitoneal injection of a portion of the extract into mice. The time from injection to the last gasping breath is recorded. The mouse unit (MU) is defined as the amount of toxin required to kill a 20 g mouse in 15 minutes. The dose in MU is estimated from the death time using the tables of Sommer (5). Since the mouse test was first reported, it has been modified, standardized and improved. Most of these modifications involved the preparation of the extract. Until

Fig. 1. Proposed structures of saxitoxin and derivatives produced by mild oxidation with hydrogen peroxide.



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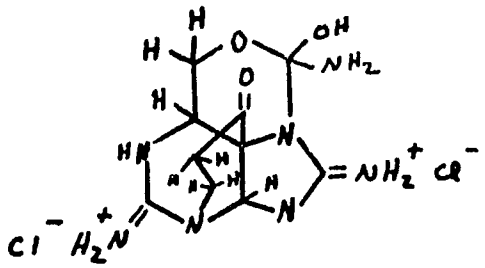

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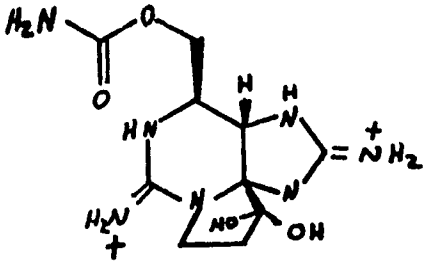

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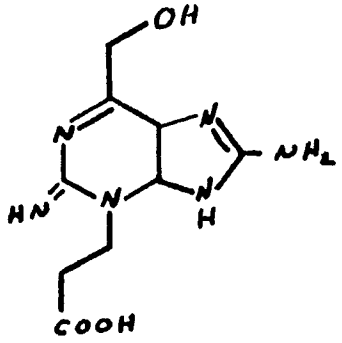

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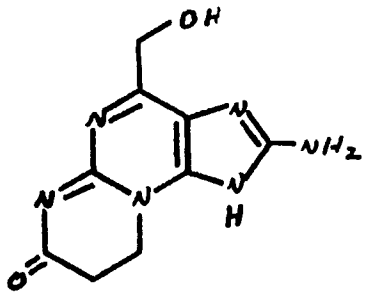


5

purified poison was available for standardization of the bioassay, results from different laboratories differed by as much as 60-70% (23). Purified saxitoxin is now used to determine a conversion factor (CF), which is obtained by dividing the number of micrograms of poison in 1 ml by the number of mouse units in 1 ml. The CF varies depending upon the particular technique and strain of mice used. The score in mouse units can be converted to ug of poison by multiplying by the conversion factor. Scores are reported as the number of ug of poison in 100 g of meat. The standard error of the mouse test is $\pm$20% of the mean (23). However, the toxicity of marginally toxic clams (80 ug/100 g) may be underestimated by as much as 60%. This may be at least partially explained by the high salt concentration in undiluted extracts, since it has been shown that NaCl reduces the recovery of added PSP by as much as 69%. Sodium acetate (0.01 M) at pH 4.0 has no affect upon bioassay results (23). The quarantine limit has been set at 80 ug (about 400 MU) of PSP per 100 grams of edible portions of shellfish meats (24).

Chemistry of PSP

Courville in Halstead (5) has compiled the most complete review of the early work on the chemistry of PSP. A mass intoxication in Wilhelmshaven, Germany, in 1885 prompted the first serious attempts to isolate the poison. Brieger (25) reported the isolation of a toxin from mussels as the gold salt which he called "mytilotoxin". A series of severe outbreaks of PSP along the California coast starting in 1927 supplied Sommer and associates at the University of California with the initiative and the source of crude material necessary for an extensive study of the chemical nature of the poison. Müller (26)

working with Sommer showed that the toxin isolated by Brieger (25) was not the toxic principle of PSP and turned to the use of ion exchangers and adsorbants rather than the precipitation reactions used by earlier workers. Müller (26) isolated a toxic preparation having an activity of about 500 MU/mg by a procedure involving: 1) extraction with acidified methanol; 2) filtration through activated carbon; 3) adsorption onto permutit and elution with potassium chloride; 4) extraction into methanol; 5) precipitation with rufianic acid; and 6) conversion to the hydrochloride. The fact that the toxin bound to the permutit, which only adsorbs strong bases, gave the first clear cut evidence that the toxin was a strong base. The toxin isolated by this procedure still contained considerable amounts of inorganic impurities. Bendien and Sommer (27), Sommer et al. (28, 29), and Riegel et al. (30) investigated the use of a number of other ion exchangers and adsorbants, and succeeded in isolating toxic preparations with specific activities as high as 1,600 MU/mg. Their basic procedure involved: 1) extraction of the toxic mussel livers with acidified ethanol; 2) decolorization with active carbon; 3) extraction of inactive material with ether; 4) cation exchange chromatography on barium Decalso (permutit); and 5) chromatography on active carbon (Norit A). Sommer et al. (28) showed that the toxicity of the poison in aqueous solution decreased with an increase in pH or temperature. Riegel et al. (30) studied the bases accompanying the poison after preliminary purification and were able to identify betaine, choline, homarine, taurine and tyrosine. Riegel et al (31) isolated a toxic preparation having a specific toxicity of 1,650 MU/mg from samples of marine plankton rich in G. catenella centrifuged from sea water

(off the California coast) in the area of a red tide. Choline and trimethylamine were also isolated.

Schantz et al. (32) reported an improved procedure for the isolation of PSP that gave a highly purified poison with better yields than the older procedures. Toxin prepared by this procedure had a specific toxicity of 5,500 $\pm$500 MU/mg and a specific optical rotation ($[\alpha]^{25}D$) of +130 $\pm$5°. Schantz (8) summarized the results of earlier work by other investigators and the results from cooperative studies beginning in 1944 involving workers at the University of California, Northwestern University, the University of Illinois, the Squibb Institute of Medical Research and the Chemical Corps Biological Laboratories at Fort Detrick, Maryland. The principal evidence for the purity of the toxin prepared by the procedure of Schantz et al. (32) as presented by Mold et al. (33) was 1) the behavior of the poison and its derivatives upon countercurrent distribution; 2) the preparation of identical material by several diverse procedures; and 3) the absence of all impurities known to be present in the crude starting extracts.

The procedure of Schantz et al. (32) involved the following steps: 1) dilute acidified ethanol extraction of ground bivalve parts mixed with a filter aid (Celite 545); 2) adsorption of the poison from the crude extract on the sodium form of Amberlite IRC-50, a carboxylic acid resin, followed by a wash with pH 4 acetate buffer which removed over 99% of the inert solids, in turn followed by fractional elution of the poison with 0.5M acetic acid; 3) chromatography on the acid form of Amberlite XE-64; 4) chromatography on acid-washed alumina in absolute ethanol. Three sources of poison were used in this study: 1) the digestive glands (hapatopancreas) of California mussels (Mytilus

californianus); 2) the siphons of Alaska butter clams (Saxidomus giganteus); and 3) the digestive glands of scallops (Pecter grandis) from the Bay of Fundy. The isolation procedure worked equally well for the poison from either mussels or clams, both of which were thought to have acquired the poison from G. catenella (34). As much as 600 pounds of siphons from 8 tons of clams, yielding 1 gram of purified poison, was used as starting material for the procedure. Attempts to isolate the poison from toxic scallops which acquired the poison from G. tamarensis (35, 36, 37, 38) failed since, although the poison was adsorbed onto the sodium Amberlite resin, it was eluted with the pH 4 acetate buffer along with the bulk of the impurities.

With minor modification, the same procedure was used to isolate the poison from axenic cultures of G. catenella (39, 40, 41). The biological, chemical and physical properties of the clam, mussel and G. catenella poisons were identical, (32, 42, 40, 33, 8, 43, 3, 39) indicating that the dinoflagellate produces the poison which is absorbed by the shellfish with no change in chemical structure. This toxin was given the name saxitoxin (STX) by Rapoport (44). Poisons very similar, if not identical, to STX have since been isolated from a Japanese crab (Zosimus aeneus) (45, 46) and have been reported to be present in the blue-green alga Aphanizomenon flos-aquae (47). The work of Alam et al. (48), however, indicates that, although the toxin of A. flos-aquae may be similar to STX, it differs in several respects.

Casselman et al. (49) described a semimicro (10-25 mg) paper chromatographic method for purification of partially purified poison and enrichment of low toxicity fractions generated during the final step of the Schantz et al. (32) procedure. This method was extended

to a preparative scale (500 mg) by the use of heavy paper (50). The poison isolated by this method had a toxicity of 5,000 - 5,800 MU/mg and appeared identical to pure saxitoxin, except that the specific rotation was only +98 $\pm 4^{o}$. Bannard and Casselman (51) demonstrated by paper electrophoresis that the low specific rotation was due to the presence of several impurities which possessed low order toxicities and little or no optical activity. Although the paper chromatographic and electrophoretic methods described by these workers are useful as a research tool and yield nearly pure STX with good recovery, they are impractical for large scale purification of saxitoxin since the paper requires extensive pretreatment to remove impurities.

Saxitoxin is a white hydroscopic solid, very soluble in water and lower alcohols and insoluble in lipid solvents. It has no ultraviolet absorption above 220 nm and appears to exist in two tautomeric forms. The Jaffe, Benedict-Behre and Weber reagents give positive color tests with the purified poison, but the Sakaguchi test is negative (40). Hydrogenation at 1 atm produces a dihydro derivative which is non toxic. The molecule contains two basic functional groups present in equivalent amounts (pK_a 8.1 and 11.5) (42, 36). Determination of the chemical structure was made enormously difficult due to the noncrystalline, highly polar and nonvolatile nature of STX. The molecular formula of the dihydrochloride has been reported as $C_{10}H_{17}N_7O_4.2HCl$ (40) or $C_{10}H_{15}N_7O_3.HCl$ (52) depending upon the drying conditions. Three structures for STX have been reported in the literature (Fig. 1). Structures 1 (53) and 2 (52) were formulated on the basis of extensive chemical and spectroscopic work. Structure 5, a pyrimido (2,1-b) purine (54) was the key degradation product leading

to the formulation of structure 2. This degradation product was formed by mild oxidation of saxitoxin with 0.8% hydrogen peroxide at 25° over a pH range of 3-12. Structure 4 is apparently the initial product of alkaline hydrogen peroxide oxidation of saxitoxin but forms structure 5 upon acid isolation (22). Structure 3 (55), the latest to be proposed, was determined from a single crystal X-ray diffraction study of the p-bromobenzene-sulfonate derivative of saxitoxin. This latest structure shows that saxitoxin is a 3,4,6-trialkyl tetrahydropurine containing three fused rings. The functional group at C 13 is described as a hydrated ketone. The dehydration of this group under vigorous drying conditions may explain the difference between the two proposed molecular formulas. The two tautomeric forms of STX seen on countercurrent distribution (33) may be the ketone and the ketone hydrate.

G. tamarensis Poison

In contrast to the enormous amount of effort expended and our knowledge of the chemical and physical properties of saxitoxin, relatively little is known about the poison produced by G. tamarensis. G. tamarensis is the dinoflagellate thought to be responsible for outbreaks of PSP along the North Sea and the North American Atlantic coast, areas ranked third and fourth, respectively, in importance where PSP is a public health hazard (7). Prior to 1972, the only areas affected along the vast Atlantic coast were the Bay of Fundy and the estuary of the Saint Lawrence River. In the late summer of 1972 a massive bloom of G. tamarensis resulted in more than 40 cases of PSP and the closing of clam flats in Massachusetts, New Hampshire and southern Maine. Outbreaks of PSP occurred again on the New England coast in the spring and summer of 1974. These recent outbreaks of PSP

in an area previously unaffected by blooms of G. tamarensis generated new interest in the chemistry of the poison produced by this armored dinoflagellate.

As previously described (8), the poison from scallops collected in the Bay of Fundy behaved differently than saxitoxin on sodium Amberlite IRC-50 resin. Two possible reasons for this difference in behavior proposed by Schantz (8) were: 1) the poisons were chemically different; or 2) impurities in the scallop extracts affected the binding properties of the resin. Experiments in which purified saxitoxin was added to scallop extracts indicated that the two toxins were chemically different, possibly having different pK_a values, since an amount of toxin equivalent to the amount of saxitoxin added was firmly bound to the Amberlite IRC-50 resin. These difficulties with scallop extracts made isolation of the poison from them impossible using the procedure of Schantz et al. (32).

Evans (56, 7) isolated two toxic fractions from mussels (Mytilus edulis) collected during an outbreak of PSP associated with a bloom of G. tamarensis off the northeast coast of Britain (57). A minor toxic fraction, purified to a specific toxicity of 1,550 MU/mg, was shown to closely resemble saxitoxin in its biological effects and behavior on the sodium and hydrogen form of Amberlite, a carboxylic acid resin. The majority of the toxicity, however, was only weakly bound to the sodium Amberlite and was eluted from the resin with pH 4 acetate buffer along with the bulk of the impurities as previously described by Schantz (8) for scallop extract. Further attempts at chromatographic separation of this weakly bound poison on Amberlite and Sephadex resins only improved the specific toxicity to 270 MU/mg

and resulted in the loss of the majority of the activity. This preparation was determined to contain at least 90% inert solids, mostly sodium chloride. Its biological effects were similar, but not identical, to saxitoxin.

Schantz (58) reported that poison from G. tamarensis had been purified in his laboratory, but gave no details on the source of the poison or the procedure used. Although no extensive studies on its chemical and physical properties had been performed, he did conclude that G. tamarensis produced a potent poison very similar to saxitoxin in its biological action, but somewhat different in its chemical and physical properties.

Schantz (3) reported that attempts to isolate the poison from axenic cultures of G. tamarensis by the method used for axenic cultures of G. catenella (40) failed, due to problems similar to those encountered with scallop extracts (8). Schantz (11) reported that although the structure of the poison from G. tamarensis had not been completely elucidated, it was different from G. catenella poison (STX).

Recently Ghazarossian et al. (59) working with Schantz, reported finding only one poison in ten year old extracts of scallop hepatopancreas collected from the Bay of Fundy. The toxin was purified by a slight modification of the procedure described by Schantz et al. (32) to a specific toxicity of 5, 150 MU/mg. On the basis of its biological activity and thin-layer chromatographic behavior, the toxin was identified as saxitoxin. These workers suggested that the freshly collected scallops may have contained structurally different toxins of a less basic character which broke down to give saxitoxin during storage.

METHODS

Mouse Test

The toxicity of aqueous solutions was determined by the intraperitoneal IP injection of one ml of solution into mice weighing approximately 20 g. Appropriate dilutions were made so that the mouse would die within 4 to 8 minutes after injection. With samples also containing acetic acid or ethanol, care was taken to insure that these compounds were not present in concentrations toxic to mice. The survival time in minutes was converted to mouse units (MU) using the tables of Sommer (5) where one MU is defined as the amount of toxin required to kill a 20 g mouse in 15 minutes. Specific toxicity is expressed as either MU/mg or MU/ug of dry material. The strain of mice used was C 57 BL/6J (Jackson Laboratories, Bar Harbor, Maine) maintained in this laboratory. Our mice had a conversion factor (CF) of 0.2.

Paper Chromatography

Paper Chromatography was run on Whatman No. 1 paper (30 x 17 cm). Standard solutions containing 1 mg/ml of the following compounds were prepared: guanidine;HCl, sulfaguanidine, creatinine, creatine phosphate, methylguanidine, guanidoacetic acid (glycocyamine), aminoguanidine sulfate, L-arginine, argininosuccinic acid, creatine hydrate, L-canavanine sulfate and streptomycin sulfate. Five or 25 µl samples were spotted along the longer dimension of the paper 2 cm from the lower edge. The paper was rolled into a cylinder, stapled and developed in a covered, cylindrical chamber at room temperature. Two solvent systems were used (P-A) n-butanol:pyridine:water (65:65:65 by volume) and (P-B) n-butanol:

HOAc:water (120:30:50 by volume). The solvent system was allowed to travel up the paper to a height of about 12 cm. The paper was removed from the chamber, airdried for 1 hour and sprayed with one of the spray reagents. R_f values were calculated as:

$$R_f = \frac{\text{Distance from origin to center of spot}}{\text{Distance from origin to solvent front}}$$

Thin-Layer Chromatography (TLC)

Pre-coated silica gel 60 plates without fluorescent indicator (EM Reagents) were stored in a desiccator and activated just prior to use at 110°C for 30 min. The plates were developed in covered rectangular glass chambers in the following solvent systems: (A) n-butanol:acetic acid:water (50:25:25); (B) tert-butanol:acetic acid:water (50:25:25); (C) ethanol:pyridine:water:acetic acid (60:40:20:10); (D) ethanol:water:acetic acid (100:40:25); (E) pyridine:ethyl acetate:water:acetic acid (75:25:30:15) (all by volume). The plates were air dried for 1 hour after development and treated with the spray reagents indicated.

Spray Reagents for Paper and Thin-Layer Chromatography

Jaffe (Picric Acid) Spray (60). The dried chromatogram was sprayed with 1% (w/v) picric acid in ethanol, dried, then sprayed with 5% ethanolic KOH.

Benedict-Behre Spray. The chromatogram was sprayed with a 1% (w/v) 3,5-dinitrobenzoic acid in ethanol, dried, then sprayed with 5% ethanolic KOH.

Weber (PCF, FCNP) Spray (61). The following stock solutions were stored at 4°C: (1) 10% (w/v) NaOH in distilled water; (2) 10%

(w/v) sodium nitroprusside (Na_2 Fe(NO) $(CN)_5 \cdot 2\ H_2O$) in distilled water; (3) 10% (w/v) potassium ferrocyanide ($K_4Fe(CN)_6 \cdot 3H_2O$). The spray solution was prepared by mixing one volume of each of the stock solutions and diluting with 3 volumes of distilled water.

Sakaguchi (62). The following stock solutions were stored at 4^oC: (1) 0.01% ∝-naphthol dissolved in ethanol containing 5% urea; (2) 100 ml of 1N NaOH containing 0.7 ml Br_2. The chromatogram was first sprayed with stock solution (1) to which KOH had been added to approximately 5% just before spraying. It was then sprayed with stock solution (2).

Diacetyl-∝-naphthol Spray (63). The stock solution stored at 4^oC was prepared by adding 20 ml of 25% (w/v)∝-Naphthol in n-propanol to 2.5 ml of aqueous 1% 2,3-butanedione (diacetyl) and diluting the mixture to 100 ml with n-propanol. The spray was prepared by mixing 1 volume of the stock solution with 1 volume of 5N NaOH just prior to spraying.

Sulfuric Acid Spray. The chromatogram was sprayed with concentrated sulfuric acid (H_2SO_4) and heated at 130^oC for 4-8 minutes.

NBD Chloride Spray (64). The chromatogram was heated at 100^oC for 15 minutes, then sprayed lightly with a fresh 1% solution of NBD-Cl (4-chloro-7-nitrobenzo-2-oxa-1,3-diazol) in methanol. The chromatogram was next sprayed heavily with 10% sodium bicarbonate ($NaHCO_3$) and heated to 110^oC to increase the color.

Hydrogen Peroxide Spray. The chromatogram was sprayed with a fresh 1% solution of H_2O_2 in distilled water and immediately heated at 100^oC for 30 min. The spots are visible under long wave UV light (360 nm).

In Situ TLC-Fluorometric Assay

Standard solutions containing 100, 80, 60, 40, 20 and 10 ug of toxin/ml were prepared from samples of major toxin H and major toxin L, isolated on the Bio-Gel column (see below) and from saxitoxin standards kindly supplied by Dr. E.J. Schantz. Standard solutions of partially purified minor toxin containing 500, 400, 300, 200, 100 and 50 MU/ml were also prepared. If only the relative amounts of the different toxins present in a particular series of samples (e.g. fractions off a column) were to be investigated, no standards were applied to the TLC plates. For the quantitative determination of the concentration of toxin in a sample, standards and test solutions were run on the same plate. All samples studied in a particular series were applied in the same volume (usually 4 μl with a 2μl Lang Levy pipet). The plates were developed in solvent system (E) for 1.5 hours at room temperature. After air drying for 1 hour, the developed plates were sprayed evenly with 1% hydrogen peroxide for 30 seconds. The plates were then heated in an oven at 100^{o}C for 30 minutes and finally desiccated over $CaCl_2$ for 1 hour. The fluorescent spots were measured in situ with a Turner Model III Fluorometer (Turner Assoc., Palo Alto, California) equipped with a TLC Scanner Door. The fluorometer was operated at the 10 X setting using the standard (110-350) lamp, a 7-60 narrow pass primary filter (peak 360 nm) and a 47 B narrow pass secondary filter (peak 436 nm). The plates were scanned at a rate of 20 mm/minute along the path of sample migration. A strip chart recorder was used to register the deflection observed.

Fluorescence Spectra

Fluorescent spectra were determined in solution with a MK-1

Sprectrofluorometer using a stabilized xenon lamp (Farrand Optical Co., New York, N.Y.).

Visible and Ultraviolet Spectra

The absorption of aqueous solutions containing 100 ug/ml of major toxin H, 100 ug/ml of major toxin L and 400 mu/ml of minor toxin were determined at 20 nm intervals from 700 to 220 nm with a Beckman DU-2 spectrophotometer.

Source of Toxin

The clams (Mya arenaria) used in this study were collected by the State of New Hampshire Fish and Game Department and the Parker River National Wildlife Refuge, Plum Island, Massachusetts, during the height of the G. tamarensis red tide that occurred along the Central New England Coast in September, 1972. The clams had scores of 2,000 - 4,000 ug of poison (as STX) per 100 g of meat. The clams were stored whole in the frozen state and thawed out just prior to processing.

Extraction and Initial Purification of the Toxins

The extraction and preliminary steps in the purification of the toxins were carried out using the procedure described by Schantz, et al. (32) with minor modifications as detailed below. Three batches of clams were carried through this procedure but only the last and largest batch (23 kg) will be described. Whole clams (23 kg) were thawed and shucked to yield meat and juice (11 kg). The meat and juice were combined, covered with 95% ethanol (1-2 l) and acidified to pH 2-3 with 1N HCl (600 ml). The clams were ground in an Oster blender and mixed with an equal volume of Celite 503. The semi-solid mixture was loosely packed into 8 Buchner Funnels (diameter 25 cm) over a pad of glass wool

and a layer of fresh Celite. Aqueous 15% ethanol acidified to pH 2-3 with conc. HCl was filtered through the funnels by gravity. The filtrate was collected and assayed periodically until only negligible activity to the mouse test remained. The filtrate (44 l) was concentrated in vacuo on a rotary evaporator. Upon standing at 4°C much material came out of solution. This material was tested and found to be inactive. The concentrate (9.2 l) was heated to 90°C, rapidly cooled in an ice bath and centrifuged at 23,000 x g at 0°C for 20 minutes. This step resulted in the removal of a large amount of inactive insoluble material with no loss of activity. The supernatant (pH 1.7) was adjusted to pH 5.7 with 1 N NaOH (2.25 l). As the pH approached 5, much material came out of solution. After adjustment to pH 5.7, the solution was again centrifuged at 23,000 x g for 20 minutes. The sediment was again tested and found to be inactive. The supernatant (11.2 l) was applied to a sodium amberlite column.

Sodium Amberlite IRC-50 Column

A 4 x 96 cm column was prepared from 1.5 kg of Amberlite IRC-50 C.P. obtained dry in the hydrogen form (Mallinckrodt Chemical Works, St. Louis, Missouri). The resin was converted to the sodium form by mixing batchwise with 1 N NaOH (1.5 l) until the pH was greater than 10. The resin was rinsed with distilled water until the pH was between 8 - 8.5 and converted back to the hydrogen form by mixing batchwise with 1 N HCl until the pH was less than 4. The resin was then rinsed with distilled water until the pH was greater than 5 and finally converted back to the sodium form by mixing batchwise with 1 N NaOH until the pH was greater than 10. The resin was again rinsed with distilled water until the pH was between 8 - 8.5 and poured into the column. The packed

column was rinsed with about 20 volumes of distilled water at a flow rate of 10 ml/minute. The discolored resin at the top of the column was removed and the sample (in 11.2 l) applied at a flow rate of 6 ml/minute. The effluent from the column was collected manually during the day in 250 or 500 ml graduated cylinders. The activity of each fraction was determined with the mouse test. At night the effluent was run into a fraction collector and 16 ml fractions were collected. These fractions were assayed periodically and pooled into larger fractions on the basis of color and activity. Application of the supernatant (42 MU/ml) was continued until the activity in the effluent rose to 20 MU/ml. At this point 6.15 l of supernatant (42 MU/ml) had been applied. The column was nearing saturation and addition of supernatant was stopped. The column was rinsed with distilled water (1.5 l) until no appreciable activity remained in the effluent. The column was then eluted with 1 M acetic acid buffered at pH 4 with a saturated sodium acetate solution (2.75 l). This removed the major toxic fraction from the column. Elution with the pH 4 buffer was continued until no more activity appeared in the effluent. The column was again rinsed with distilled water (2.05 l) and then eluted with 0.5 M acetic acid. This removed the minor toxic fraction from the column. The column was finally eluted with 1 N HCl. One run of the sodium Amberlite column required about 3 days. The sodium form of the resin was regenerated and the remainder of the supernatant (5.1 l) applied. The major and minor toxic fractions were isolated by repeating the process.

Purification of Minor Toxin

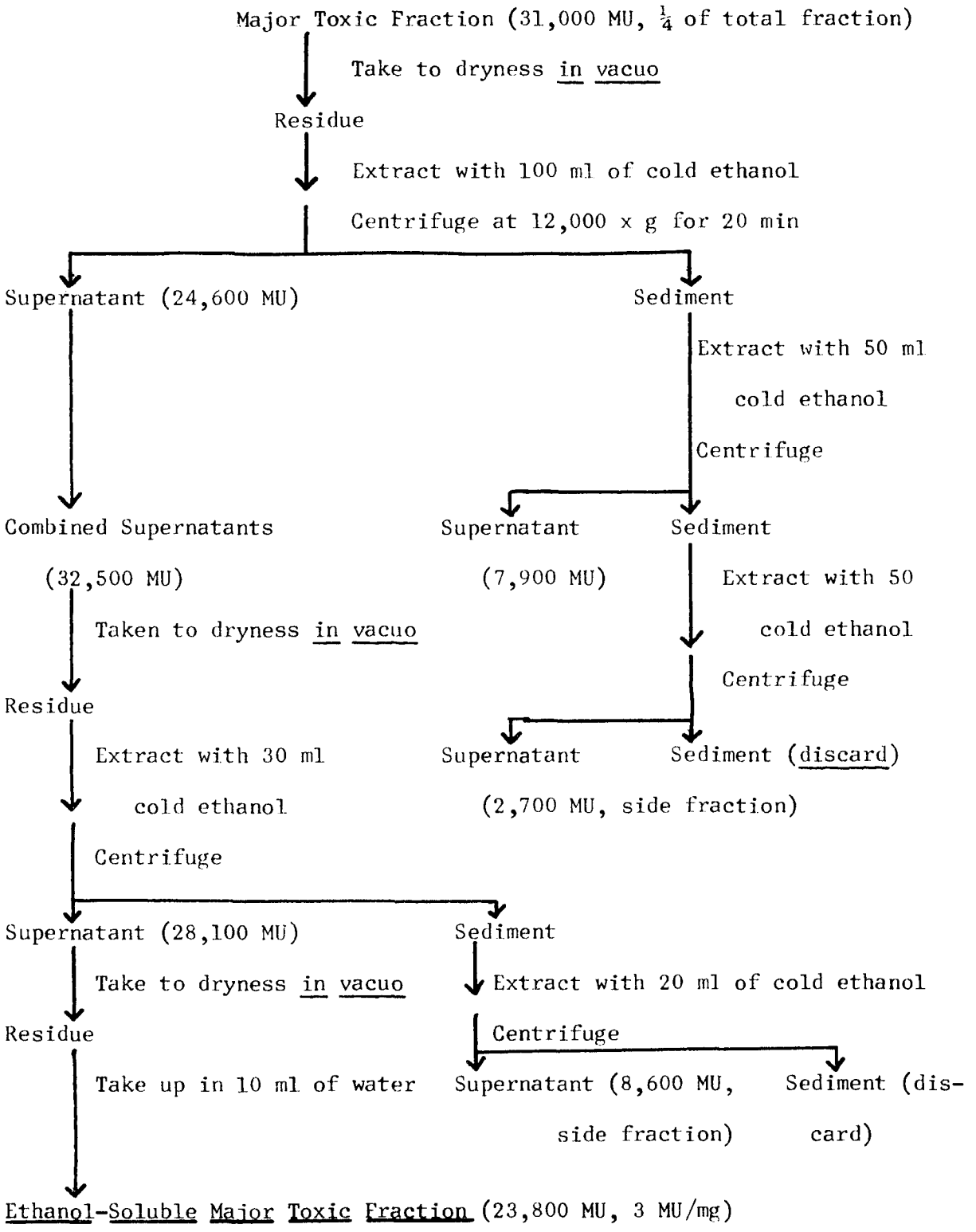
The minor toxic fraction was purified by two passes through an Amberlite CG-50 (200-400 mesh) column (2 x 43 cm) in the hydrogen

form according to the procedure of Schantz *et al.* (32) for STX to yield the *minor toxin* as described below. The combined minor toxic fraction from the Amberlite IRC-50 column (above) was concentrated *in vacuo* on a rotary evaporator to a concentration of about 1,000 MU/ml (volume about 100 ml). The Amberlite CG-50 resin (a finely divided form of Amberlite IRC-50) was prepared by conversion to the sodium form, washing with distilled water, reconversion to the hydrogen form and washing with distilled water until the pH was greater than 4. The sample was applied at a flow rate of 40 ml/hr with a peristaltic pump. Fourteen ml fractions were collected. After sample application had been completed, the column was rinsed with 250 ml of deionized distilled water, then eluted with 0.1 M acetic acid. Every tenth tube was assayed with the mouse test until the peak of activity was located and then every tube was assayed. The active fractions were combined, concentrated to a volume of 70 ml and applied to a fresh Amberlite CG-50 column prepared by the same procedure. The column was washed with deionized distilled water and again eluted with .1 M acetic acid, 14 ml fractions being collected. The peak of activity was located by assaying every other tube with the mouse test. Tubes containing activity were combined and lyophilized to give the purified *minor toxin* and the weight determined.

Purification of the Major Toxic Fraction

The *major toxic fraction* from the Amberlite IRC-50 column was divided into four equal batches (1.4 l each) and each batch processed in the following manner (Fig. 2). The sample was concentrated on a rotary evaporator to about 200 ml, the pH brought down to 5 with concentrated HCl (about 25 ml) and the fraction taken to dryness. The residue was dried further for 24 hours in a vacuum desiccator contain-

Fig. 2. Diagram for the isolation of the Ethanol-Soluble Major Toxic Fraction



ing calcium chloride lumps, sodium hydroxide pellets, and concentrated sulfuric acid in separate dishes. A pale yellow crystalline solid was obtained which was extracted with 100 ml of cold ethanol. The mixture was centrifuged at 12,000 x g for 20 minutes. The pellet remaining after pouring off the supernatant was re-extracted with 50 ml of cold ethanol and the mixture again centrifuged. The supernatants were combined, taken to dryness *in vacuo*, and the residue desiccated for 24 hours. The residue was extracted with 30 ml of cold ethanol and the mixture centrifuged. The supernatant was concentrated *in vacuo* to dryness and the residue taken up in 10 ml of water to give the *ethanol-soluble major toxic fraction* which was immediately applied to the Bio-Gel column (4 x 100 cm) (BG 4-1 to 4, Table 6).

Bio-Gel (4 x 100 cm) Column

A Bio-Gel column (4 x 100 cm) was prepared by pouring 400 g of Bio-Gel P - 2 (200-400 mesh) (Bio-Rad Laboratories, Richmond, California) as a slurry into a 4 x 104 cm column and washing with deionized distilled water at a flow rate of 84 ml/hour. Fourteen ml fractions were collected and evaluated on the basis of total activity, solids content and appearance in the *in situ* TLC-fluorometric assay. Variable amounts of toxicity which were bound to the column were eluted with 0.1 M acetic acid. Between each run, the column was washed with 2 l of 0.1 M acetic acid and rinsed with at least 4 l of deionized distilled water.

Bio-Gel (2.6 x 90 cm) Column

A (2.6 x 100 cm) glass column was packed with Bio-Gel P-2 (200-400 mesh) to give a bed height of 90 cm. Samples from the first Bio-Gel column dissolved in 0.1 M acetic acid were applied through a

flow adapter and eluted with 0.1 M acetic acid at a flow rate of 40 ml/hour. Five ml fractions were collected.

The effluent from the column was monitored using the mouse test and by spotting 4 ul samples from each fraction onto a 5 x 6 cm TLC plate. Up to 20 fractions were evaluated per plate. Without development in any solvent system, the plate was sprayed directly with 1% H_2O_2 heated at 100°C for 30 minutes and observed under a long wave UV lamp (360 nm). Fractions giving fluorescent spots were further evaluated using the TLC-fluorometric procedure described above.

RESULTS

Extraction and Initial Separation of the Major and Minor Toxic Fractions

A summary of the weight and activity of fractions at different stages in the isolation procedure described in the Experimental section is shown in Table 1. From 23 kg of clams, 11 kg of meat and shell liquor were obtained after shucking. These yielded a total of 44 l of acid alcoholic extracts which after two centrifugation steps had a total activity of 472,000 MU. Because of the limited size of the sodium Amberlite IRC-50 column, the concentrated supernate (11 l) had to be applied in two runs. Summaries of the weight and activity of fractions from the first and second runs on the Amberlite IRC-50 column are shown in Tables 2 and 3 respectively. The greater part of the activity was eluted from the sodium Amberlite IRC-50 column with pH 4 acetate buffer and is therefore referred to as the major toxic fraction. Acetic acid (0.5 M) eluted another toxic fraction, which is referred to as the minor toxic fraction.

Isolation of Minor Toxin

Fraction no. 20 (Table 2) from the first sodium Amberlite IRC-50 column and fractions no. 14-16 (Table 3) from the second were pooled, concentrated in vacuo and applied to a hydrogen Amberlite CG-50 column. The activity was eluted as a single peak (141,000 MU) with 0.1 M acetic acid. The active fraction was concentrated in vacuo and applied to a second hydrogen Amberlite column. Again the activity was eluted as a single peak with 0.1 M acetic acid. Table 4 shows the weight and

TABLE 1. Weight and Activity of Various Fractions from the Isolation of the Paralytic Shellfish Poisons.

	Weight	Activity	Specific Toxicity
	(gm)	(MU)	(MU/mg)
Whole clams	23,000	---	---
Meat and juice	11,000	---	---
Aqueous 15% Ethanol Extract			
First centrifugation			
Sediment	353	---	---
Supernatant	---	527,000	---
Second centrifugation			
Sediment	105	---	---
Supernatant	786	472,000	0.6
Sodium Amberlite columns			
pH 4 Acetate Buffer Elution			
(Major Toxic Fraction)	---	162,300	---
.5 M Acetic Acid Elution			
(Minor Toxic Fraction)	---	108,000	---

TABLE 1. (cont'd)

Isolation of Minor Toxin			
Minor Toxic Fraction	9.2	106,000	11.5
First Hydrogen Amberlite Column	0.736	141,000	192
Second Hydrogen Amberlite Column			
Fraction no. 20-26 (Minor Toxin)	0.065	89,000	1,380
Fraction no. 17-19, 27-29.	0.047	20,600	430
Bio-Gel Column	---	---	2,040
Isolation of Major Toxins			
Major Toxic Fraction	282	124,000	0.44
Ethanol Soluble MATF	30.9	117,700	3.8
Major Toxin	---	87,000	1,400-3,200
Major Toxin H	---	---	1,300-1,800
Major Toxin L	---	---	2,300-4,200

TABLE 2. Activity of Fractions from the First Sodium-Amberlite IRC-50 Column Run.

Operation	Fraction Number	Volume	Activity		Recovery
		(L)	(MU/ml)	(MU)	(%)
Sample		6.15	42.0	258,300	
Sample Application	1	2.4	0	0	
(Fractions obtained	2	1.0	2.0	2,040	
during this process)	3	1.0	5.2	5,160	
	4	1.0	7.8	7,840	
	5	.25	9.2	2,300	
	6	.25	12.5	3,100	
	7	.25	22.8	5,700	
Total		6.15		26,140	10.2
First Water Rinse	8	.25	24	5,950	
	9	.25	21.5	5,370	
	10	.25	19	4,750	
	11	.50	8.6	4,300	
	12	.25	9.0	2,260	
Total		1.5		22,630	8.8
Elution with pH 4	13	.25	0	0	
Acetate Buffer	14	.40	9.6	3,800	
(Major Toxic	15	1.95	48	95,600	
Fraction)	16	.05	0	0	
Total		2.75	38.5	99,400	38.5
Second Water Rinse	17	.3	9.0	2,700	
	18	1.75	1.22	2,140	
Total		2.05		4,840	1.9

TABLE 2. (cont'd)

Elution with .5 M	19	.92	0	0	
Acetic Acid	20	2.3	24	62,700	
(Minor Toxic	21	.75	2.0	1,800	
Fraction)					
Total		3.97		64,500	25.0
Elution with 1 N HCl	22	2.0	0	0	
Total Activity Recovered				217,500	84.2

TABLE 3. Activity of Fractions from the Second Sodium Amberlite IRC-50 Column Run.

Operation	Fraction Number	Volume	Activity		Recovery
		(L)	(MU/ml)	(MU)	(%)
Sample		5.1	42	214,000	
Sample Application	1	5.1	4.0	20,000	9.5
First Water Rinse	2	1.0	12	12,000	
	3	.5	8.3	4,200	
	4	.5	C	0	
Total		2		16,200	7.7
Elution with pH 4	5	.35	10	3,400	
Acetate Buffer	6	.25	9.5	2,380	
(Major Toxic	7	.5	9.65	4,830	
Fraction)	8	2.82	18.5	52,300	
	9	.25	0	0	
Total[a]		4.18		62,900	29.4
Second Water Rinse	10	1.5	0	0	0
Elution with .5 M	11	.5	0	0	
Acetic Acid (Minor	12	.25	0	0	
Toxic Fraction)	13	.25	0	0	
	14	.25	5.6	1,400	
	15	.4	17.5	7,000	
	16	1.4	25	35,100	
Total		3.05		43,500	20.3
Total Activity Recovered				143,000	66.9

[a] The fraction collector malfunctioned during elution of this fraction, resulting in the loss of some activity.

TABLE 4. Weight and Activity of Active Fractions Eluted from the Second Hydrogen Amberlite CG-50 Column

Fraction Number	Total Solids	Activity		Specific Toxicity
	(mg)	(MU/ml)	(MU)	(MU/mg)
15	31.9			
16	30.9	22.4	300	10
17	20.9			
18	15	606	8,500	566
19	12.7			
20	9	805	11,300	1,253
21	9.9			
22	7.8	1,500	21,000	2,692
23	8.82			
24	6.51	1,315	18,400	2,829
25	6.37			
26	4.48	764	6,500	1,449
27	6.09			
28	4.97	99	1,400	279
20-26 [a]	64.64		89,400	1,383
17-19, 27-29 [a]	47		20,600	436

[a] These values are for pooled fractions.

activity of fractions from the second hydrogen Amberlite CG-50 column. The most active fraction (no. 24) had a specific toxicity of 2,829 MU/mg. Fractions no. 20-26 were pooled. This material, referred to as minor toxin, had a specific toxicity of 1,383 MU/mg. Side fractions no. 17, 18, 19, 27, 28 and 29 were pooled (20,600 MU, 436/MU/mg), taken to dryness in vacuo and applied to a Bio-Gel P-2 (4 x 100 cm) column in 10 ml of water. The activity was bound to the column and could not be eluted with deionized distilled water. The activity was eluted as a single peak with 0.1 M acetic acid. This treatment brought the specific toxicity of the best fractions up to 2,300 MU/mg. TLC of both of these preparations of minor toxin showed predominantly one component (Fig. 3).

Isolation of Major Toxin

Fraction no. 15 from the first sodium Amberlite IRC-50 column run (Table 2) and fraction no. 8 from the second run (Table 3) were combined and found to have a total activity of 124,000 MU. This material (0.44 MU/mg) was divided into four portions and each portion was purified to a level of 3-4 MU/mg with negligible loss of activity by a series of extractions into cold ethanol. The values shown in parentheses in Fig. 2 were obtained from a typical run through the ethanol extraction procedure. The weight and activity of the ethanol soluble major toxic fraction (ethanol soluble MATF) is shown in Table 5. The fifth run through this procedure was a work up of side fractions obtained from the first four runs.

Each portion of the ethanol soluble MATF was run separately on the large diameter Bio-Gel column (4 x 100 cm) (BG 4-1 to 5, Table 6). Fig. 4 shows the distribution of activity eluted in a typical run of

Fig. 3. Thin-layer chromatography of saxitoxin and the G. tamarensis poisons on silica gel plates. A,B,C,D, indicate solvent systems (see Methods section). 1,2,3, indicate saxitoxin, major toxin, and minor toxin, respectively. Plates were sprayed with sulfuric acid and charred.

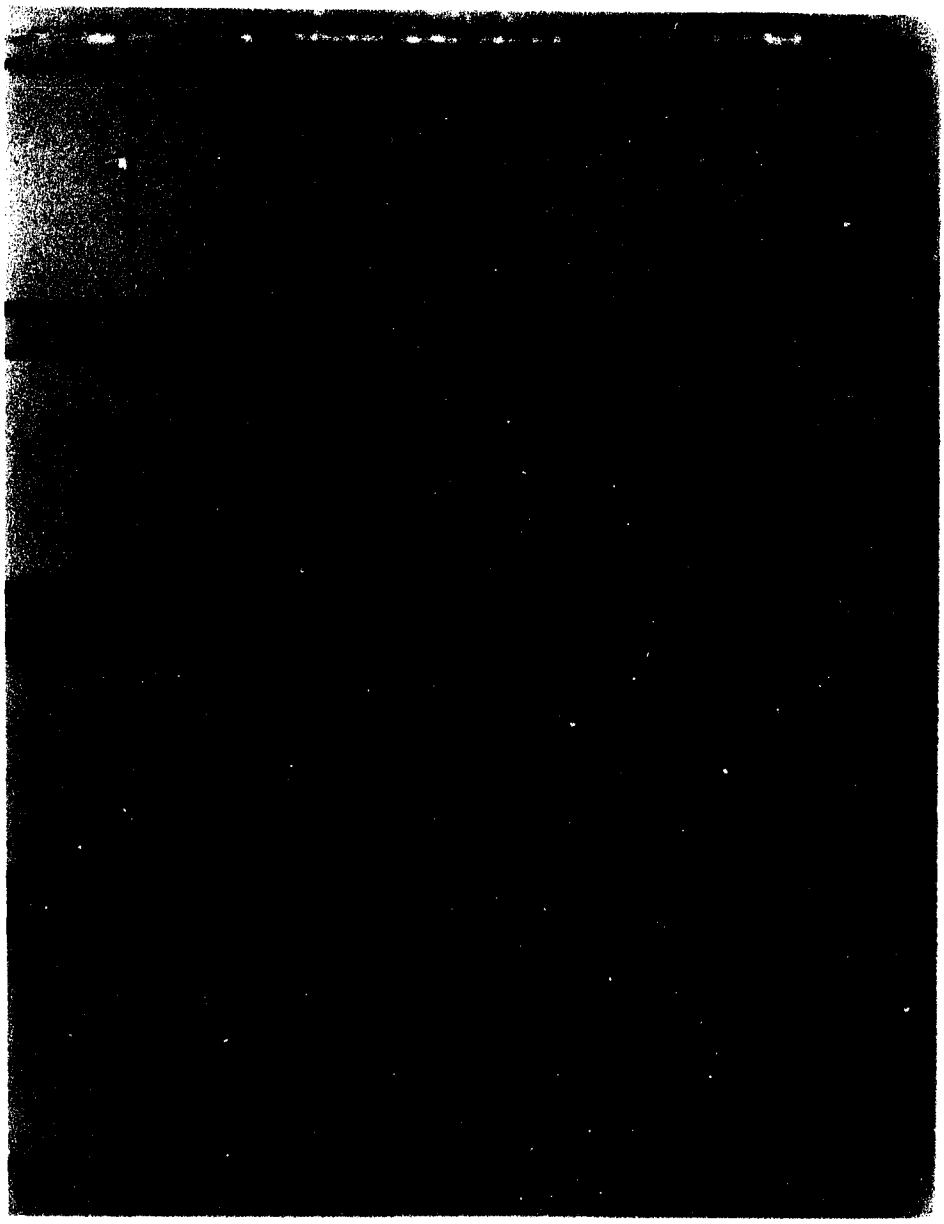


TABLE 5. Weight and Activity of Ethanol Soluble Major Toxic Fraction (Ethanol Soluble MATF)

Run Number	Weight	Activity	Specific Toxicity
	(g)	(MU)	(MU/mg)
1	7.5	22,100	2.96
2	5.1	20,100	3.94
3	8.0	23,800	2.98
4	5.3	20,400	3.82
5 [a]	5.0	31,300	6.25
Total Ethanol Soluble Activity		117,700	(94.9% Recovery)

[a] The starting material for this extraction was the pooled side fractions from the first four extractions (numbers 1-4).

TABLE 6. Weight and Activity of Sample and Pooled Fractions for Individual Runs on the Large Diameter (4 x 100 cm) Bio-Gel Column.

Sample and Eluant		Tube Number	Activity (MU)	Weight (mg)	Specific Toxicity (MU/mg)	Recovery (%)
BG4-1	Ethanol Sol.					
	MATF no. 1		22,100	7,500	2.94	
	Water Elution	86-89	14,800	6.83	2,170	
		84,85,90,91	4,500	6.19	730	
	Total		19,300			87
BG4-2	Ethanol Sol.					
	MATF no. 2		20,100	5,100	3.94	
	Water Elution	76-80	800			
		102-105	12,720	5.96	2,130	
		100,101,106	4,420	1.91	2,310	
	Total		17,940			89
BG4-3	Ethanol Sol.					
	MATF no. 3		23,800	8,000	2.98	

TABLE 6. (cont'd)

	Water Elution	75-80	440			
		98-99	320			
		100-106	4,330			
		107-110	1,020			
	Total		6,110			26
	0.1M Acetic Acid					
	Elution	75-79	1,760	9.46	190	
		80	1,480	5.94	250	
		81	6,400	5.81	1,100	
		82	2,980	2.55	1,130	
		83-84	1,190	.63	1,890	
	Total		13,720			58
BG4-4	Ethanol Sol.					
	MATF no. 4		20,400	5,300	3.85	
	Water Elution	75-80	520			
		101-109	16,100	10.95	1,470	
		98-100, 110-112	933			
	Total		17,550			86

TABLE 6. (cont'd)

BG4-5	Ethanol Sol.				
	MATF no. 5		31,300	5,000	6.25
	Water Elution	96[a]	620	.32	
		97[a]	2,220		
		98	3,700	1.56	2,415
		99	4,620		
		100	4,300		
		101	3,100		
		102	2,250		
		103	1,670		
		104	860		
	Total		23,410		75
BG4-7 - BG4-4		101-109	16,100	10.95	1,470
	Water Elution		0		
	0.1M Acetic Acid	79-81	2,130	2.82	754
	Elution	82	2,770	1.85	1,500
		83	4,350	2.43	1,790

TABLE 6. (cont'd)

	84-85	6,000	3.20	1,880	
	86-87	1,170	1.22	957	
Total		16,420			102
BG4-8 - BG4-5	100,102	4,150	2.2	1,890	
Water Elution		0			
0.1M Acetic Acid	75-78[b]	1,110			
Elution	79-83[c]	3,920			
Total		5,030			121
BG4-9 - BG4-8	79-83	3,920			
BG4-7	101,103	2,430			
Total		6,350			
0.1M Acetic Acid	95-98[b]	1,000			
Gradient Elution	99-104[c]	3,650			
Total		4,650			73

[a] Tubes #96 and 97 were pooled and shown by TLC to be pure major toxin H, specific toxicity 1,800 MU/mg.

[b] Predominately major toxin H.

[c] Predominately major toxin L.

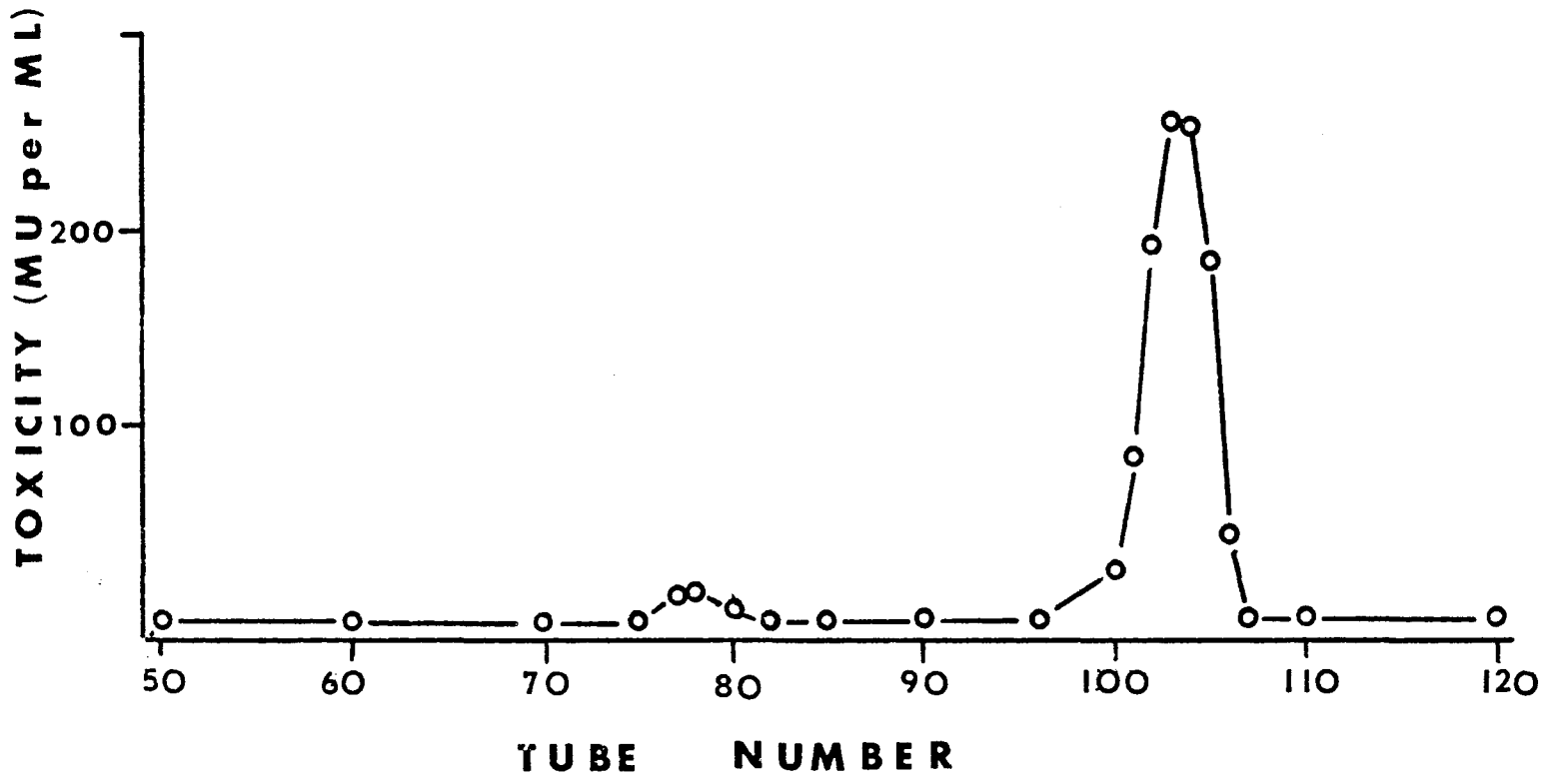


Fig. 4. Toxicity of fractions collected during a typical run of the ethanol soluble major toxic fraction on the large diameter Bio-Gel column (BG4-2). The column was eluted with deionized distilled water.

the ethanol soluble MATF on the Bio-Gel column. Table 6 lists the weight and activity of the sample and pooled fractions for each run on the large diameter Bio-Gel column. Generally, after application of ethanol soluble MATF to the column, two peaks of activity were eluted with deionized distilled water. The first peak contained only about 5% of the total activity and was not studied further. The second peak contained about 80% of the activity applied to the column. Conductivity measurements and weight determinations indicated that this main peak was eluted from the column after the majority of salts and other inert material. For some unknown reason, the majority of activity in the third sample of ethanol soluble MATF (BG4-3) was bound to the column and was not eluted with water but was subsequently eluted with 0.1 M acetic acid. The recovery of activity from the first four runs on the Bio-Gel column ranged from 86.5 to 89.2%. Fractions from the main peak of activity eluted with deionized distilled water having a specific toxicity greater than 2,000 MU/mg are referred to as major toxin.

Upon a second passage of a portion of major toxin through a Bio-Gel (4 x 100 cm) column, the activity remained bound to the packing and could not be eluted with deionized distilled water. The activity was eluted from the column in one sharp peak with 0.1M acetic acid (BG 4-7, Table 6, which is a rerun of fractions 101-109 from run BG4-4). The second passage through the Bio-Gel column did not significantly improve the specific toxicity of the sample. The removal of salts from the toxin appears to affect its behavior on Bio-Gel columns. That the separation is not strictly a gel filtration process is indicated by the fact that the salts were eluted from the column before the toxin. The binding of certain organic groups to polyacrylamide gels has been

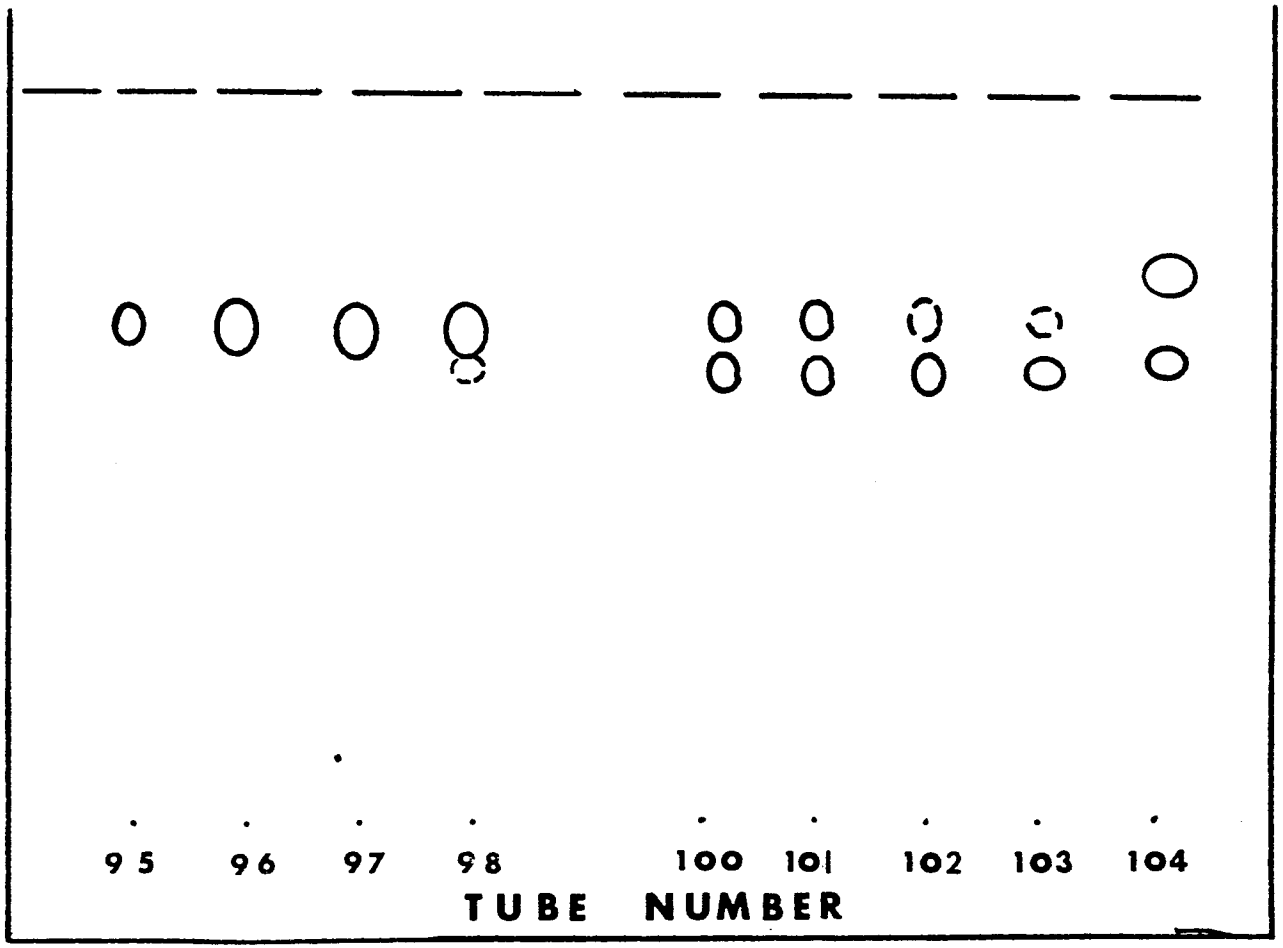
previously reported (65, 66). The binding appears to be affected by the ionic strength of the medium.

TLC of major toxin in solvent systems A, B, C and D showed an intense green spot when sprayed with H_2SO_4 and charred (Fig. 3). In solvent systems A, B and C a much less intense yellow spot was observed just below the green spot. Both spots were scraped from the plates, the scrapings extracted with water and the extracts injected into mice. Both extracts killed mice within 7 minutes. The high R_f component was given the name major toxin H and the low R_f component major toxin L. No other spots were observed under visible or long wave UV light after spraying plates with the following reagents: Jaffe, Benedict-Behre, Weber, Sakaguchi, diacetyl-∝-napthol, H_2SO_4 and NBD-Cl.

Separation of Major Toxins H and L

In order to determine if there was any separation of the two components of major toxin on the Bio-Gel (4 x 100 cm) column, sample no. 5 (Table 5) of the ethanol soluble MATF was applied to the column (BG4-5, Table 6). The fractions were assayed with the mouse test and a single peak of activity was observed. Active fractions were lyophilized and the residues weighed and taken up in distilled water adjusted to pH 4.0 with HCl. The individual concentrated fractions were run on TLC in solvent system C. The developed plate (Fig. 5) clearly showed that major toxin H was eluted slightly ahead of major toxin L. Fractions containing both major toxin H and L were combined, lyophilized and rechromatographed on the Bio-Gel column (BG4-8, Table 6). This time the activity was bound to the column and subsequently eluted with 0.1M acetic acid. Since the TLC-fluorometric assay for PSP was developed about this time, the activity was located by spotting each frac-

Fig. 5. TLC pattern of fractions from the large diameter Bio-Gel column (BG4-5) in solvent system C. The plate was sprayed with 1 % hydrogen peroxide and heated. The spots indicate fluorescent zones under long wave UV light. The upper spot at fraction 104 is where quenching was observed.


95
96
97
98
100
101
102
103
104
TUBE NUMBER

tion, using a 6 x 10 cm TLC plate as a spot plate, and spraying with H_2O_2 and heating. Fractions giving a fluorescent spot were further evaluated using the in situ TLC-fluorometric assay. Again, major toxin H was eluted just prior to major toxin L (Fig. 6). Fractions containing a mixture of major toxin H and L from runs BG4-7 and BG4-8 were again combined, lyophilized and applied to the Bio-Gel column (BG4-9, Table 6). This time the column was eluted with a linear gradient of acetic acid, 0 - 0.1M and again only partial separation was observed.

Samples of the poison previously run on the large diameter Bio-Gel column and lyophilized were dissolved in 5 ml of 0.1 M acetic acid and applied to a smaller diameter Bio-Gel column (2.6 x 90 cm). The column was eluted with 0.1M acetic acid. Table 7 lists the weight and activity of the sample and pooled fractions for several runs on the small diameter Bio-Gel column. This smaller diameter column gave somewhat better separation of major toxin H and major toxin L. By pooling and rechromatographing fractions rich in one toxin or the other on the small diameter Bio-Gel column, both major toxins H and L were isolated (Fig. 7, 8 and 9).

The H_2O_2-Fluorescence Reaction

Heating the paralytic shellfish poisons in the presence of hydrogen peroxide, both in solution and on TLC plates, was found to induce the formation of fluorescent derivatives. About 25 different solvent systems were tried before one was found which was suitable for the quantitative determination of major toxin H, major toxin L and saxitoxin (or G. tamarensis minor toxin) on a single TLC plate. Mixtures of the three poisons gave three distinct fluorescent spots after TLC in solvent system E, treatment with H_2O_2 and heating (Fig.

Fig. 6. The elution of major toxin H and major toxin L from the large diameter Bio-Gel column (BG4-8, Table 6). The peak height was determined using the standard *in situ* TLC-fluorometric assay. The column was eluted with deionized distilled water followed by 0.1 M acetic acid.

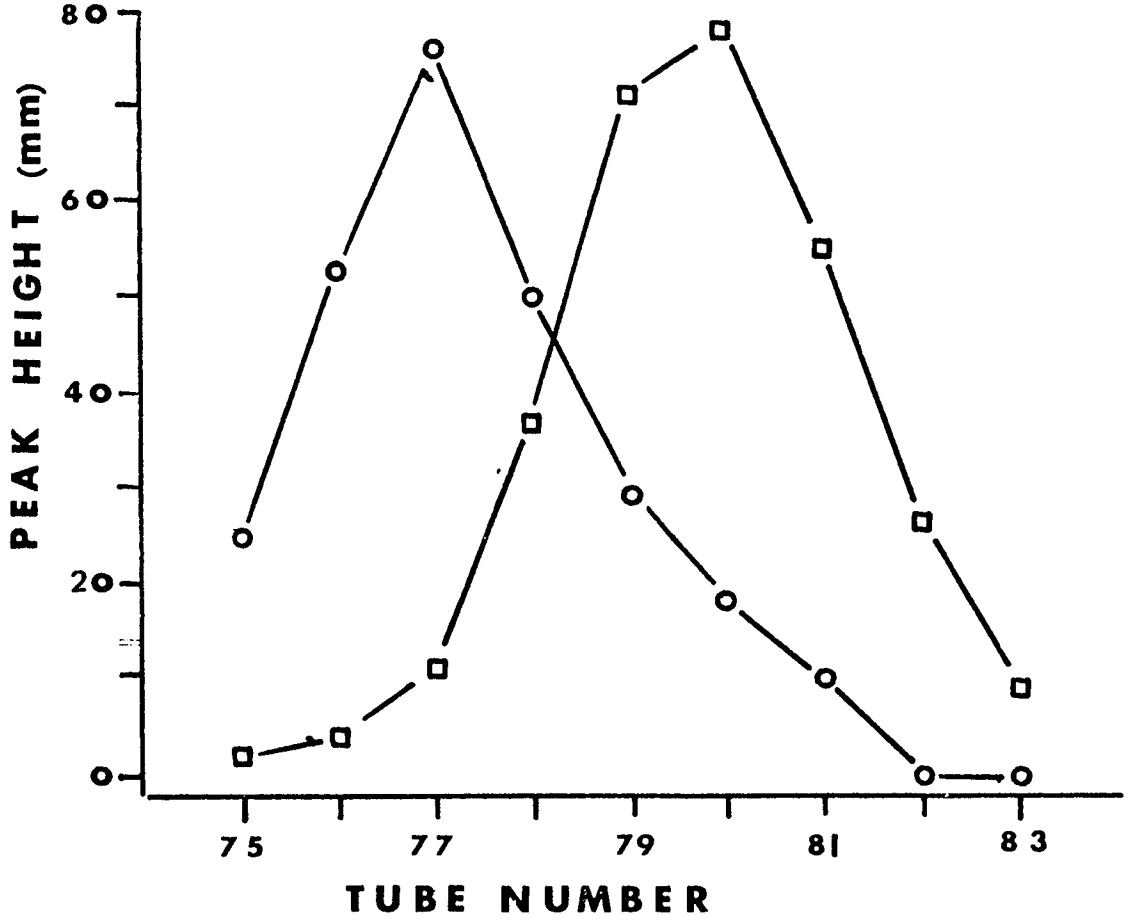

PEAK HEIGHT (mm)
80
60
40
20
0
75
77
79
81
83
TUBE NUMBER

TABLE 7. Weight and Activity of Sample and Pooled Fractions for Several Runs on the Small Diameter (2.6 x 90 cm) Bio-Gel Column.

Sample and Eluant		Tube Number	Activity (MU)	Recovery (%)
BG-2.6-1	BG4-5	98-99[f]	7,250	
	BG4-8	75-78	1,120	
	BG4-9	95-98	1,000	
Total Sample			9,370	
0.1M Acetic Acid		93-99[a]	4,450	
Elution		100-101[a]	1,570	
		102-106[b]	2,260	
		107-110[c]	1,180	
Total Recovered			9,460	101
BG-2.6-2	BG4-2	102-105[f]	6,150	
		86-89	9,700	
Total Sample			15,850	
0.1M Acetic Acid		88-92[a]	2,410	
Elution		93-99[b]	6,400	
		100-103[c]	2,000	
		104-108[d]	1,500	
Total Recovered			12,310	78
BG-2.6-3	BG2.6-1	107-110	1,180	
	BG4-9	99-104	3,650	
	BG2.6-2	100-103	2,000	
Total Sample			6,830	

TABLE 7. (cont'd)

0.1M Acetic Acid	89-92[b]	1,030	
Elution	93[c]	860	
	94[c]	960	
	95-101[e]	3,740	
Total		6,590	96

---

[a] Pure Major Toxin H.

[b] Predominately Major Toxin H

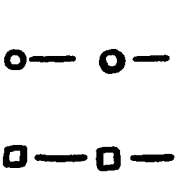
[c] Predominately Major Toxin L

[d] Pure Major Toxin L, specific toxicity 2,333 MU/mg.

[e] Pure Major Toxin L, specific toxicity 4,200 MU/mg

[f] Remainder of sample used. A portion of the original sample had been used for other purposes.

Fig. 7 Elution of major toxins H and L from the small diameter Bio-Gel column (BG2.6-1, Table 7). The column was eluted with 0.1 M acetic acid.



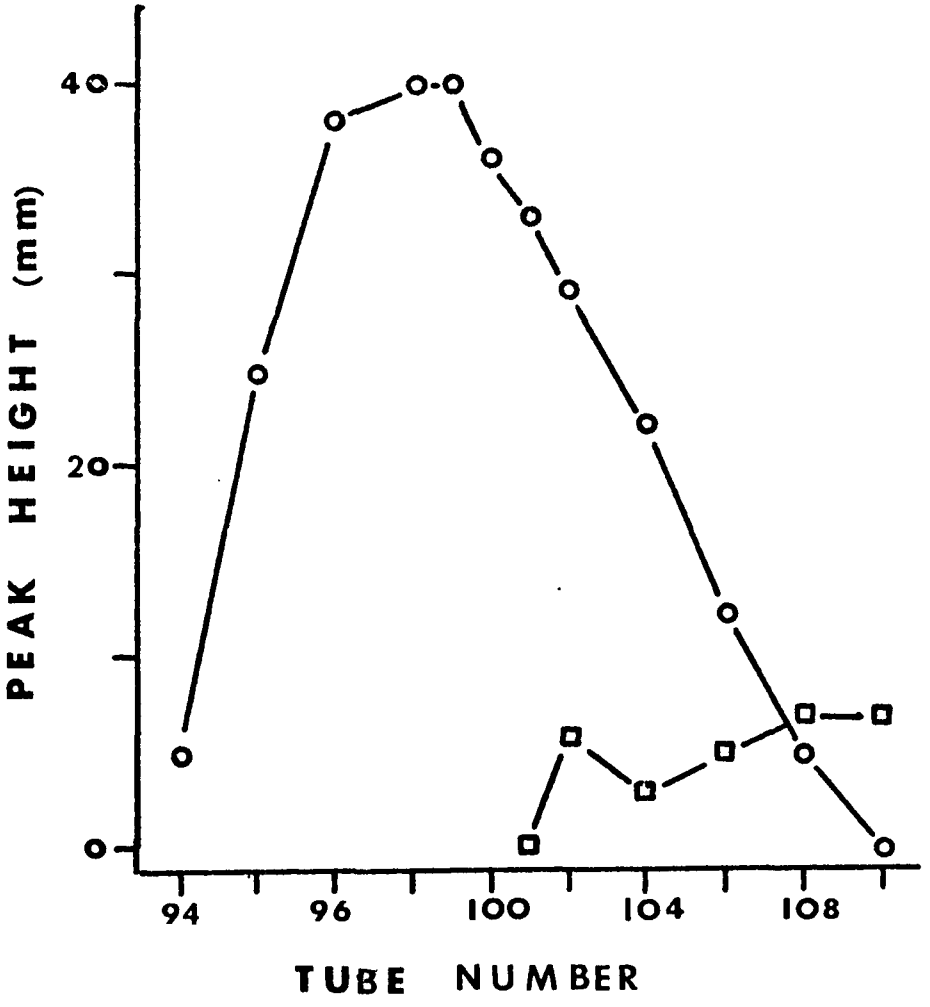
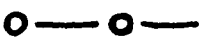

PEAK HEIGHT (mm)
40
20
0
94
96
100
104
108
TUBE NUMBER

Fig. 8. The toxicity and elution pattern of major toxins H and L in fractions from the small diameter Bio-Gel column (BG 2.6-2). The column was eluted with .1 M acetic acid.

activity in Mouse Units per ml determined with the mouse test.



peak height (mm) for major toxin H

peak height (mm) for major toxin L

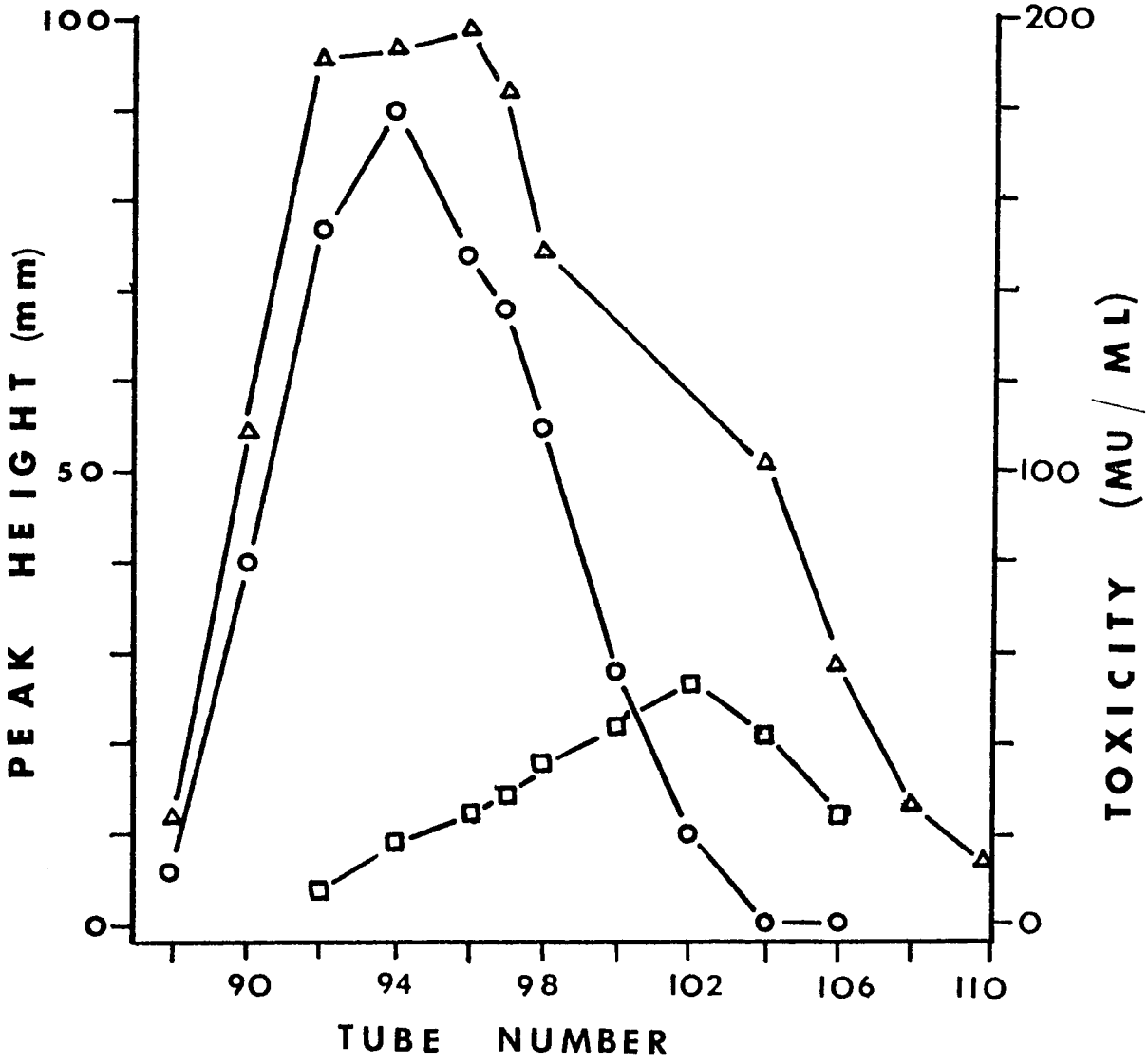
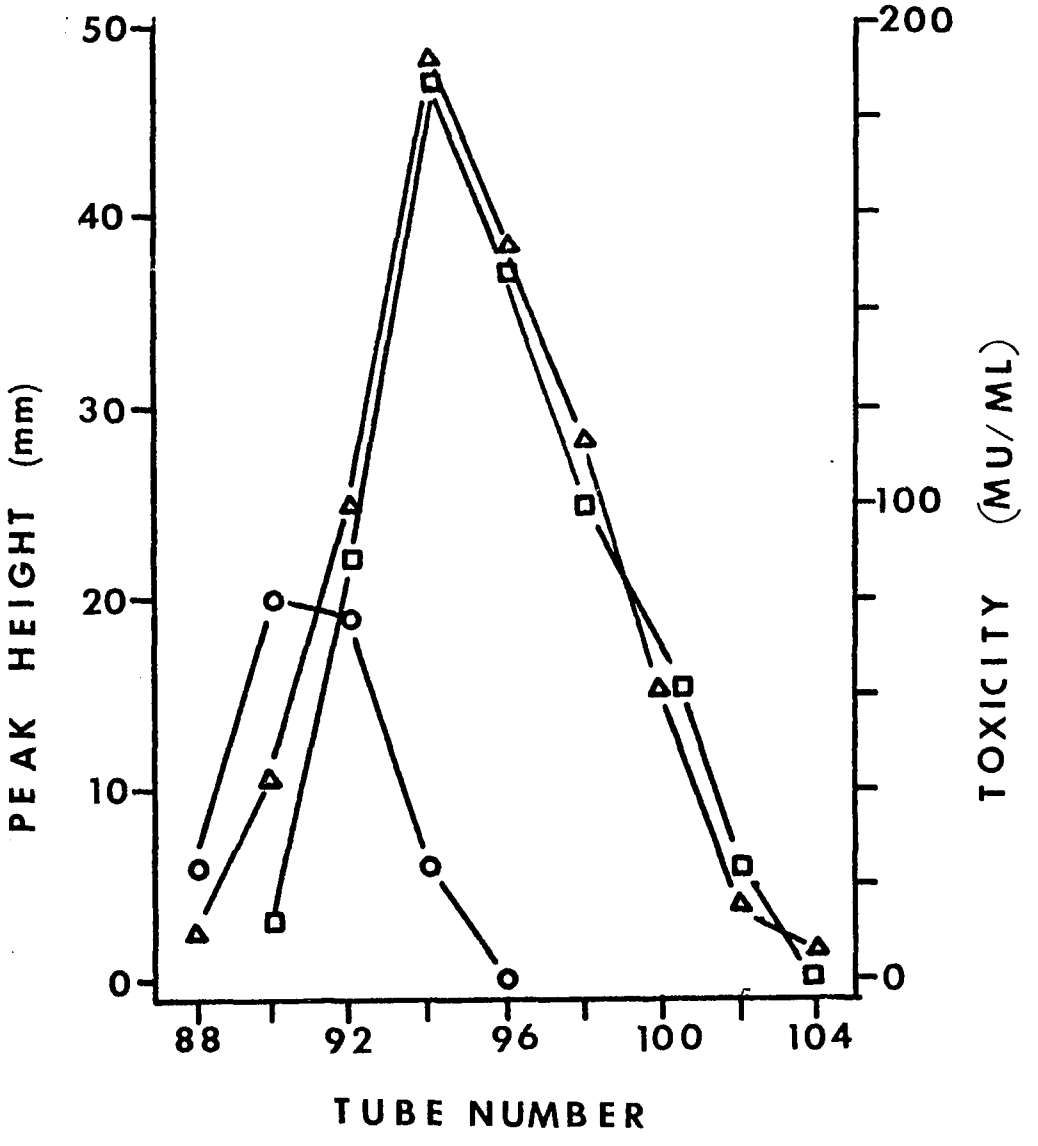

100
50
0
200
100
0
PEAK HEIGHT (mm)
TOXICITY (MU / ML)
90
94
98
102
106
110
TUBE NUMBER

Fig. 9. The toxicity and elution pattern of major toxins H and L in fractions from the small diameter Bio-Gel column (BG2.6-3, Table 7). The column was eluted with 0.1 M acetic acid.

Toxicity in Mouse Units per ml determined with the mouse test.

peak height for major toxin H

peak height for major toxin L


50
40
30
20
10
0
PEAK HEIGHT (mm)
200
100
0
TOXICITY (MU/ML)
88
92
96
100
104
TUBE NUMBER

10) (Table 8). Under long wave UV light the spots appeared light blue against a dark background. The three fluorescent spots showed up as individual peaks when scanned with the Turner Fluorometer (Fig. 11). In all solvent systems tested, mixtures of saxitoxin and G. tamarensis minor toxin traveled as a single spot. In order to determine the specificity of the H_2O_2-fluorescence reaction, a number of compounds were tested (Table 9). Only proline, tyrosine and streptomycin sulfate reacted to give fluorescent spots. The intensity of these spots upon scanning with the fluorometer, however, was less than 1/100 of the intensity given by an equal amount of PSP at the wave lengths used.

When the TLC plates were treated with hydrogen peroxide prior to development, each toxin, including the STX standard, gave two fluorescent spots (Fig. 12) (Table 8). The excitation and emission maxima of these separated fluorescent derivatives fell into two groups. For each toxin tested, the higher R_f derivative had a lower wavelength excitation and emission maximum (excitation 330-334 nm; emission 380-386 nm) than the lower R_f fluorescent derivative (excitation 360-362 nm; emission 412-417 nm). The fluorescent derivatives of STX and G. tamarensis minor toxin and major toxin H and major toxin L all appeared to be non-toxic when injected into mice.

Quantitative Measurements

A standard mixture of STX and major toxin was used to determine the effect of several variables on the intensity and stability of the fluorescent derivatives. Table 10 shows the effect of spraying time on peak height. Figure 13 shows the effect of heating time. A spraying time of 30 seconds with 1% H_2O_2 and heating at 100° for 30 minutes

Fig. 10. TLC pattern in solvent system E of (a) saxitoxin, (b) G. tamarensis minor toxin, (c) saxitoxin plus minor toxin, (d) G. tamarensis major toxin H, (e) major toxin H plus major toxin L, (f) G. tamarensis major toxin L, and (g) a mixture of saxitoxin, minor toxin, and major toxins H and L. The spots were observed under long wavelength UV light after spraying with H_2O_2 and heating.

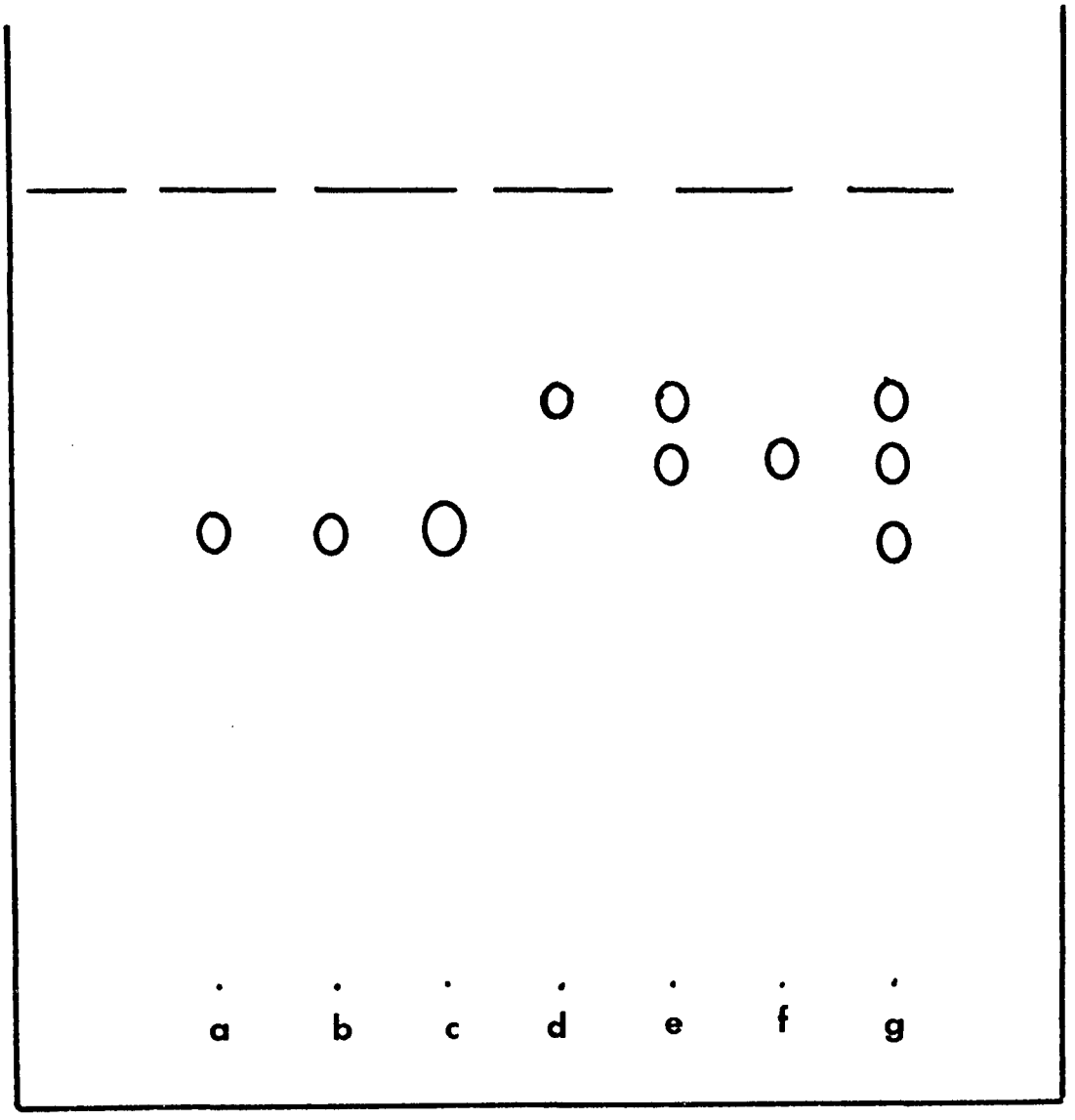

a
b
c
d
e
f
g

TABLE 8. R_f Values of the Toxins and their Fluorescent Derivatives[b] with Excitation and Fluorescence Maxima.

Compound	R_f[a]	Excitation (nm)	Emission (nm)
Saxitoxin	.58		
Low R_f derivative	.29	360	415
High R_f derivative	.75	330	383
Minor Toxin	.56		
Low R_f derivative	.28	360	412
High R_f derivative	.74	332	384
Major Toxin H	.74		
Low R_f derivative	.15	360	415
High R_f derivative	.57	333	380
Major Toxin L	.68		
Low R_f derivative	.15	362	417
High R_f derivative	.56	334	386

[a] TLC was run in solvent system (E).

[b] The fluorescent derivatives were obtained by running TLC plates as previously described with the exception that after sample application the plates were sprayed with H_2O_2, heated, and desiccated for 1 hr, producing the fluorescent derivatives prior to development in solvent system (E). After development, the fluorescent spots were scraped off the plates and the scrapings extracted with 2 ml of water adjusted to pH 4 with HCl, and the extracts spun in a clinical centrifuge. The fluorescence spectra of the supernates were determined.

Fig. 11. Scans of plates spotted with samples of the paralytic shellfish poisons and developed using the standard *in situ* TLC-fluorometric assay.

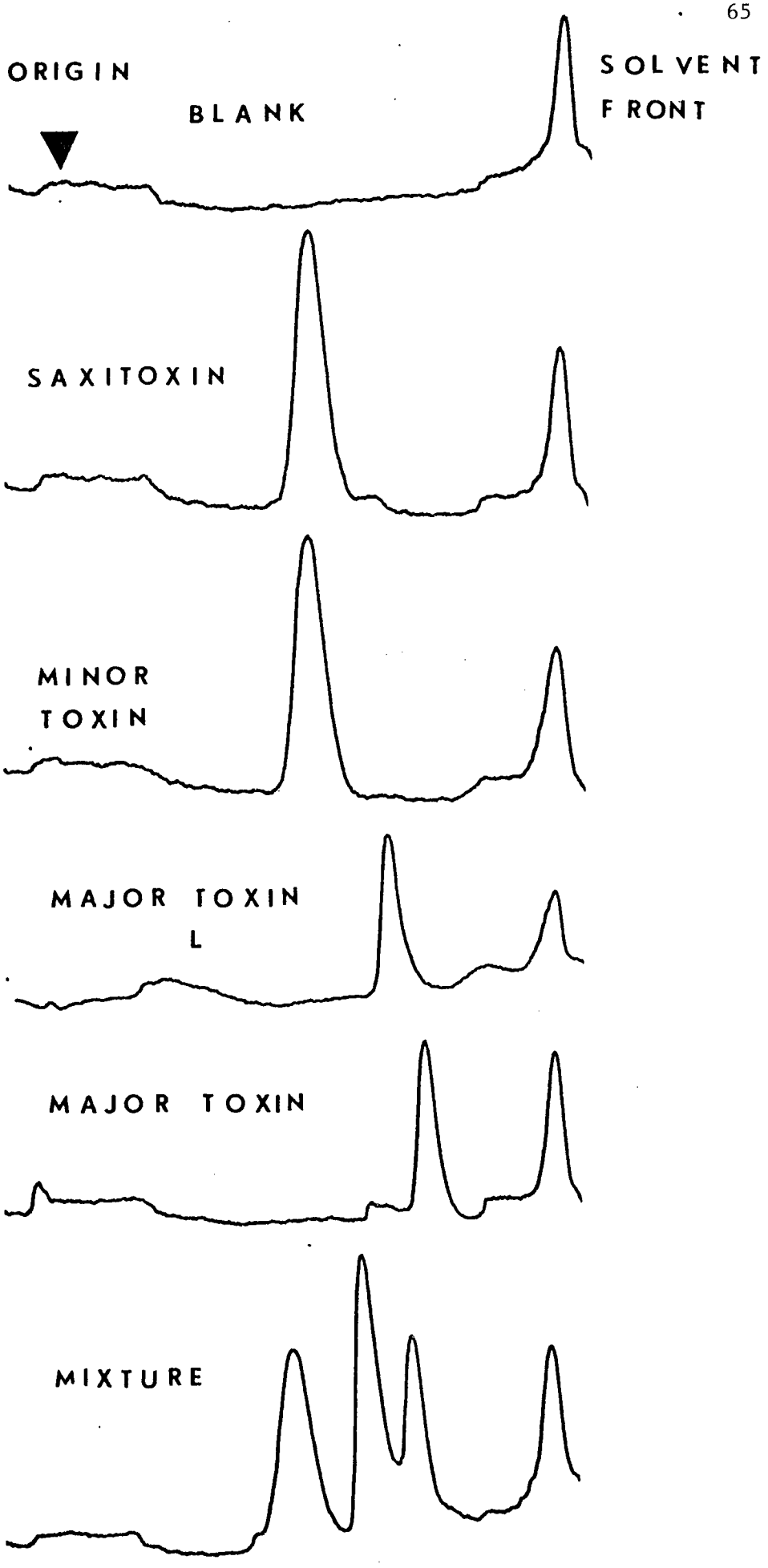

ORIGIN
BLANK
SOLVENT
FRONT
SAXITOXIN
MINOR
TOXIN
MAJOR TOXIN
L
MAJOR TOXIN
MIXTURE

TABLE 9. Specificity of H_2O_2-Fluorescence Reaction for PSP[a]

	No Fluorescence	Fluorescence
Amino Acids:	Glutamic acid	Proline
	Aspartic acid	Tyrosine
	Asparagine	
	Alanine	
	Arginine	
Purine and Pyrim-	Uracil	
idine Bases:	Guanine	
Guanidine	Guanidine	Streptomycin
Derivatives:	sulfaguanidine, creatinine	sulfate
	creatine phosphate	
	methylguanidine	
	guanidoacetic acid	
	aminoguanidine sulfate	
	creatine hydrate	
	phosphocreatine	
	argininosuccinic acid	
	canavanine sulfate	

[a] The above compounds were tested at 5 ug/spot.

Fig. 12. TLC pattern of the H_2O_2-fluorescent derivatives of the paralytic shellfish poisons in solvent system E. The plates were spotted with the poisons, sprayed with 1% H_2O_2, heated and desiccated for 1 hour; then developed in solvent system E. The spots were observed under long wavelength UV light. a, b, c, d indicate saxitoxin, minor toxin, major toxin H and major toxin L, respectively.

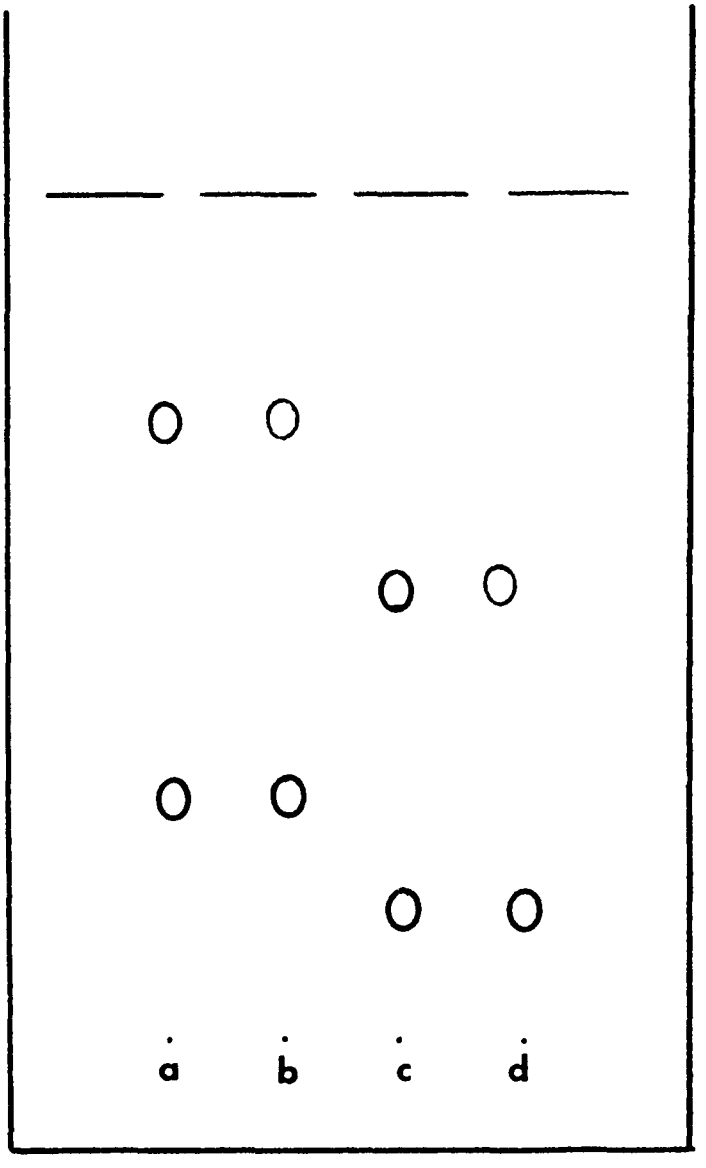

a
b
c
d

TABLE 10. The Effect of Spraying Time on Peak Height [a]

Sample	Spraying Time (seconds)			
	15	30	45	60
	Peak Height (mm)			
STX	35	49	52	49
Major Toxin H	145	141	144	126
Major Toxin L	94	94	97	86

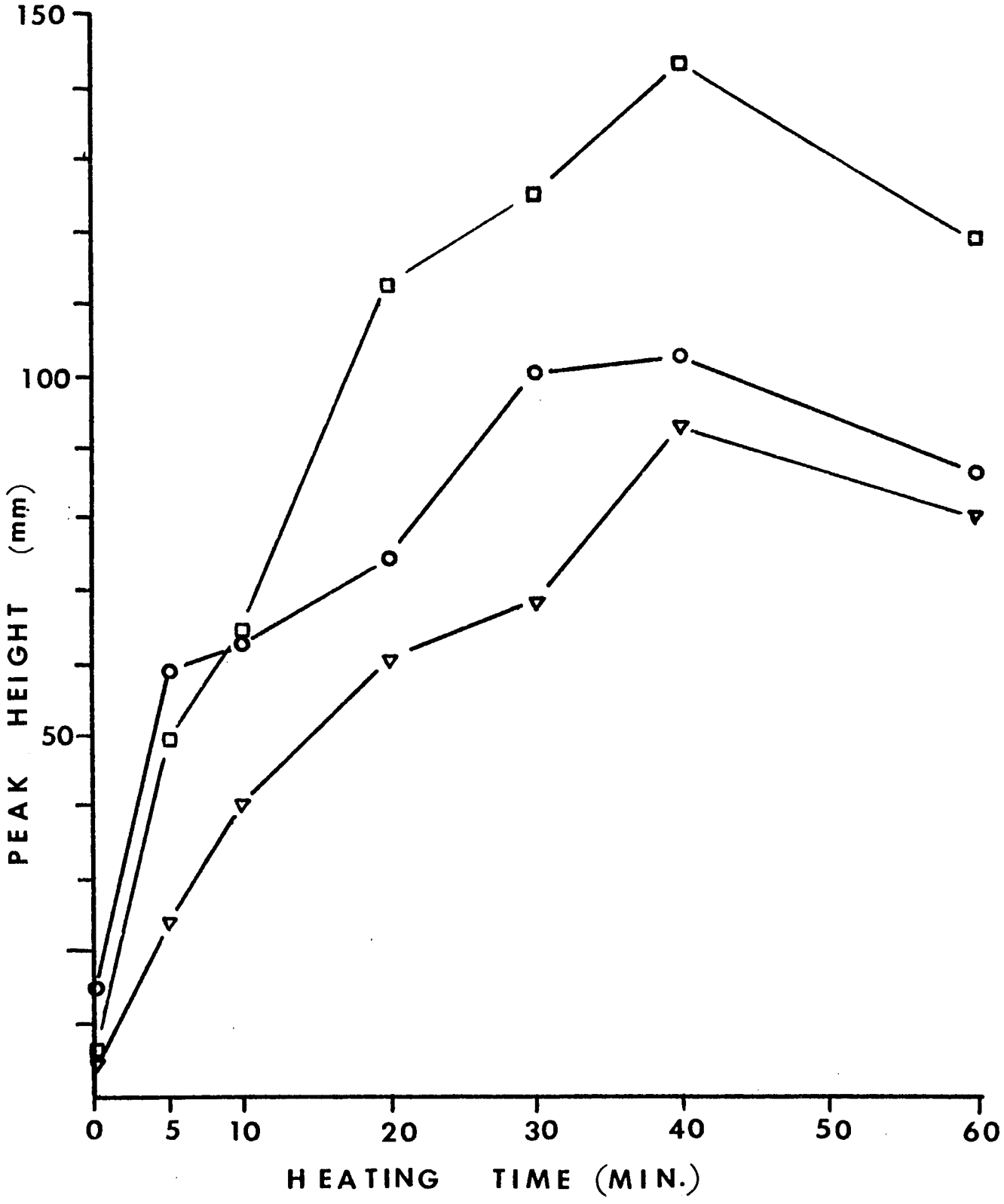
[a] All the values shown were obtained from a single 20 x 20 cm TLC plate. Four sets of two spots each containing a standard mixture of STX, Major toxin H and Major toxin L were applied to the plate. The plate was processed by the standard procedure except that the plate was sprayed with 1% H_2O_2 for a total of 60 seconds. A glass plate was moved across the plate at 15 second intervals to give the spraying times indicated.

Fig. 13. The effect of heating time on peak height. Each point represents the average peak height of 4 spots on two different 5 x 20 cm TLC plates. The plates were spotted with a standard mixture of saxitoxin, major toxin H and major toxin L and processed by the standard procedure for the *in situ* TLC-fluorometric assay except that the plates were heated at 100^0 C for the times indicated.

▽—▽— saxitoxin

○—○— major toxin H

□—□— major toxin L


150
100
50
PEAK HEIGHT (mm)
0
5
10
20
30
40
50
60
HEATING TIME (MIN.)

were found to give optimum results. The stability of the fluorescent derivatives and the effect of desiccation are shown in Table 11. After desiccation for 1 hour, the intensity of the fluorescent spots remained essentially constant for at least 24 hours. Repeated scans of the same plate did not affect the peak heights observed. Using the standard procedure for the in situ TLC-fluorometric assay described in the methods section, five duplicate samples containing 0.24 ug of saxitoxin standard gave a mean peak height of 33.2 ± 0.7 mm and five duplicate samples containing 0.24 ug of major toxin II gave a mean peak height of $33.4 \pm .92$ mm.

For all the toxins tested, there appeared to be a linear relationship between the peak height and the amount of toxin applied between 40 and 400 ng of toxin per spot (Fig. 14, 15). Above 400 ng per spot, the fluorescence began to fall off, possibly due to quenching.

After major toxin II and major toxin L had been isolated and standard solutions prepared, it was possible to determine the concentration of all three paralytic shellfish poisons present in a solution using the in situ TLC-fluorometric assay. Multiplying the concentration in ug/ml, determined from the graph, by the specific activity in MU/μg gives the number of MU/ml. This value can be compared with the value obtained using the mouse test (Table 12). Good agreement was observed between the fluorometric assay and the mouse test for fractions off the Bio-Gel column and partially purified preparations of G. tamarensis minor toxin.

Physical and Chemical Properties of the G. tamarensis Poisons

Purified preparations of minor toxin, major toxin II and major toxin L when injected into mice produced symptoms identical to those

TABLE 11. The Stability of the Fluorescent Derivatives with Time and the Effect of Desiccation [a]

Undesiccated

Major Toxin H µg	Time (hours) 0	1	4	24
	Peak Height (mm)			
.1	14	12	11	12
.2	24	22	21	22
.3	31	27	27	29
.4	38	35	34	36
.5	45	42	42	43

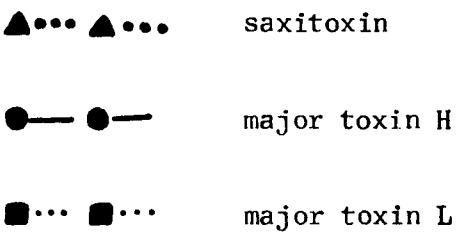
Desiccated

Major Toxin H µg	Time (hours) 0	1	4	24
	Peak Height (mm)			
.1	9	10	10	10
.2	22	20	20	22
.3	30	30	28	31
.4	38	37	37	39
.5	47	46	44	47

TABLE 11. (cont'd)

[a] Two major toxin H standard curves were spotted on a single 20 x 20 cm TLC plate. The plate was processed by the standard procedure except that after removal from the oven, the plate was cut in two. One half of the plate was desiccated for 1 hour over calcium chloride and then scanned at the time intervals indicated. The other half of the plate was scanned immediately, ommitting the desiccation step, and again at the time intervals indicated.

Fig. 14. Typical calibration curves for saxitoxin and G. tamarensis major toxins H and L using the standard in situ TLC-fluorimetric assay.



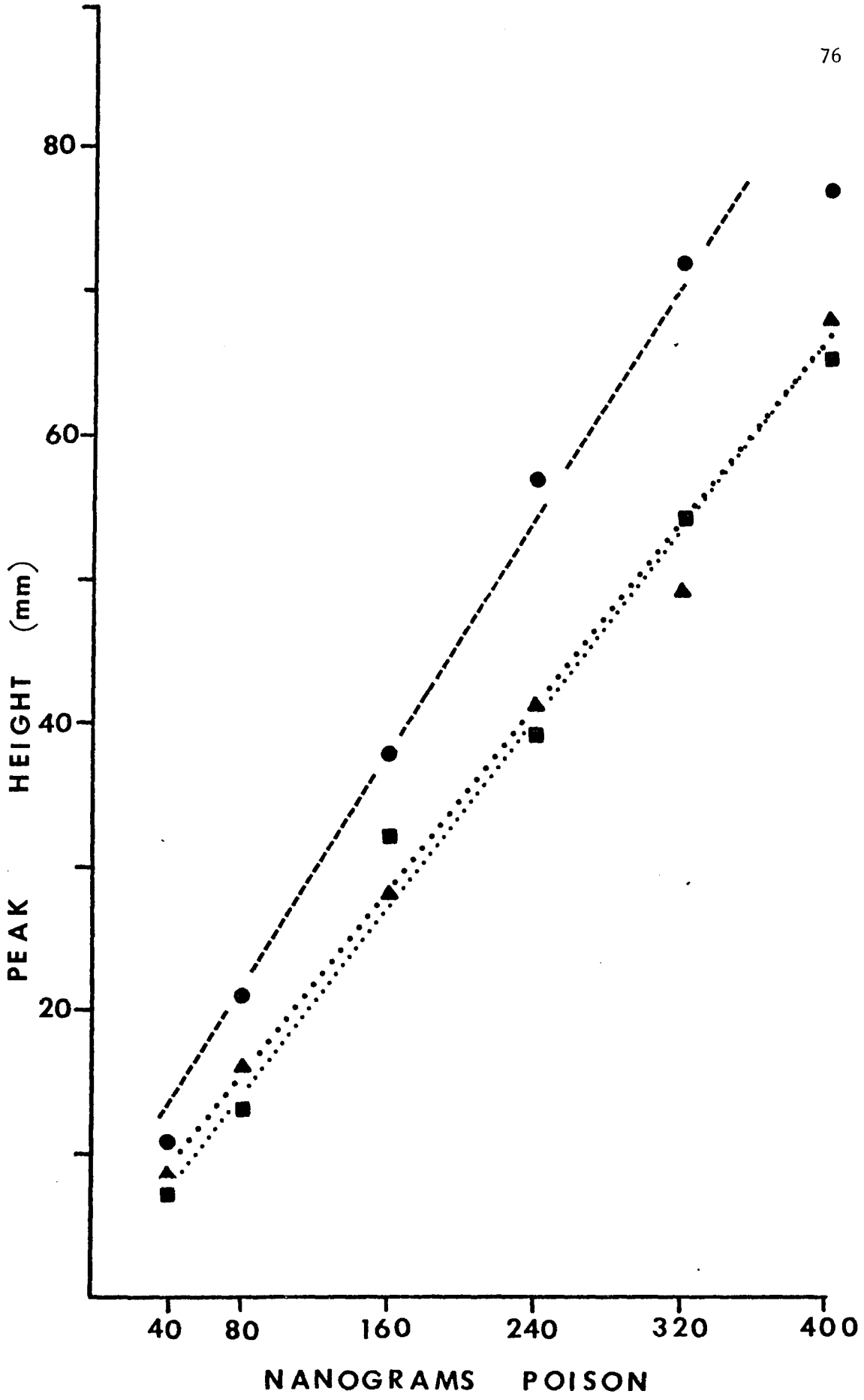

80
60
40
20
PEAK HEIGHT (mm)
40
80
160
240
320
400
NANOGRAMS POISON

Fig. 15. Typical calibration curve for G. tamarensis minor toxin using the TLC-fluorimetric assay.

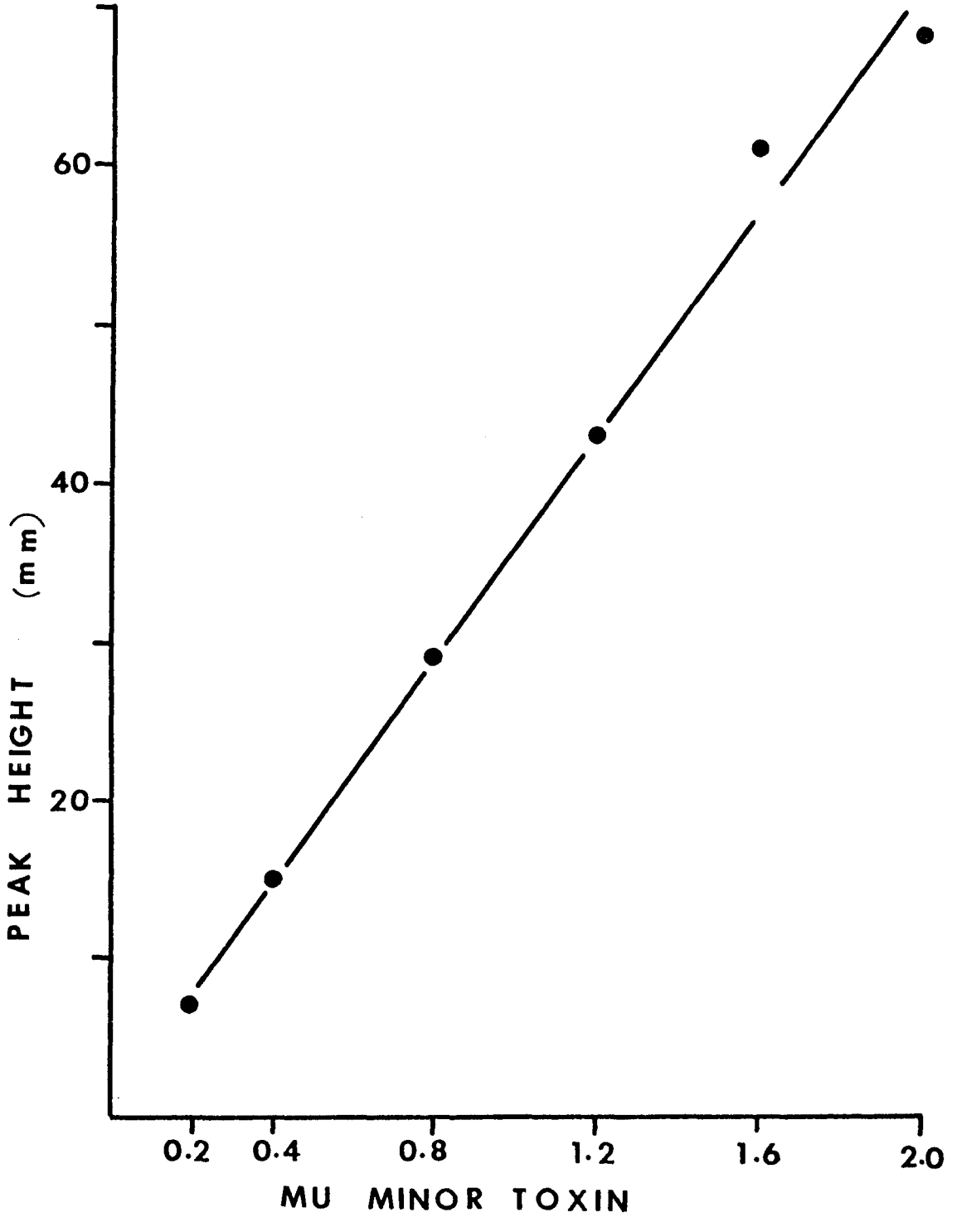

60
40
20
PEAK HEIGHT (mm)
0.2
0.4
0.8
1.2
1.6
2.0
MU MINOR TOXIN

TABLE 12. Estimation of Toxin Concentration and Activity Using the *In Situ* TLC-Fluorometric Assay and Comparison with Values Determined with the Mouse Test.

Sample No. [a]	In Situ TLC-Fluorometric Assay				Mouse Test
	conc. ug/ml			MU/ml [b]	MU/ml
	MA H	MA L	STX		
1	-	-	-	-	-
2	8	-	-	14	24
3	65	-	-	117	109
4	113	-	-	203	192
5	126	-	-	226	194
6	70	4	-	143	198
7	62	8	-	146	184
8	11	8	-	100	127
9	-	24		101	102
10	-	-	98.1	543	500
11	-	-	18.3	100	100

[a] Samples 1 through 9 were fractions from a typical run of major toxin on a Bio-Gel column. Samples 10 and 11 were partially purified preparations of minor toxin.

[b] Calculated by multiplying μg MA H/ml by 1.8, μg MA L/ml by 4.2, and μg STX by 5.5 (See text).

of STX, namely, nervousness, ataxia, convulsions, respiratory distress, paralysis and death, usually within eight minutes, depending on the dose. G. tamarensis major and minor toxins also produce symptoms similar to STX (67, 68, 69) when injected (intra gas bladder) into killifish (Fundulis heteroclitus), namely, sectoral darkening of light adapted fish and death.

Lyophilized samples of all three G. tamarensis toxins were light, fluffy white solids which rapidly picked up water when exposed to the air, making accurate weighing difficult. All three toxins were soluble in water and lower alcohols and insoluble in lipid solvents. Major toxin L appeared to be less soluble in cold ethanol than major toxin H since it appeared to be concentrated in the insoluble side fractions obtained during the ethanol extraction procedure. No ultraviolet or visible absorption above 220 mu was observed for the G. tamarensis poisons (Table 13).

The specific toxicity of three different preparations of major toxin H was determined. The values obtained were 1,800 MU/mg; 1,383 MU/mg; and 1,278 MU/mg. The specific toxicity of two preparations of major toxin L was determined. The values obtained were 2,333 MU/mg and 4,220 MU/mg. Although care was taken to avoid possible sources of error, the small quantities available and the hydroscopic nature of the poisons made accurate weight determinations very difficult, even when the balance and samples were placed in a dry glove box.

TLC of STX and the G. tamarensis major and minor toxins in four different solvent systems is shown in Fig. 4. The color reactions given by STX and the major and minor toxins after TLC in solvent A and treatment with different spray reagents are listed in Table 14. In

TABLE 13. UV-Visible Spectrum of the G. tamarensis Toxins.

Absorbance[a]	Major Toxin L	Major Toxin H	Minor Toxin
(nm)	100 ug/ml	100ug/ml	400 MU/ml
700	.00	.00	.00
680	.00	.00	.00
660	.00	.00	.00
640	.00	.00	.00
620	.00	.00	.00
600	.00	.00	.00
580	.00	.00	.00
560	.00	.00	.00
540	.00	.00	.00
520	.00	.00	.00
500	.00	.00	.00
480	.00	.00	.00
460	.00	.00	.00
440	.00	.00	.00
420	.00	.00	.00
400	.00	.00	.00
380	.00	.00	.00
360	.00	.00	.00
340	.00	.01	.00
320	.01	.02	.01
300	.02	.03	.03
280	.03	.04	.04
260	.05	.07	.06
240	.07	.09	.10
220	.74	.56	.82
215	1.46	1.09	1.43

[a] The absorbance was determined using 100 ul quartz cells and a Beckman DU-2 spectrophotometer.

TABLE 14. Color Reactions of STX and the G. Tamarensis Major and Minor Toxins with Different TLC Spray Reagents.[a]

Spray	STX[b]	Major Toxin H[c]	Major Toxin L[c]	Minor toxin[b]
Jaffe (picric acid)	Orange	Neg.	Neg.	Orange
Benedict-Behre (3,5-dinitrobenzoic acid)	Purple	Neg.	Neg.	Purple
Weber (ferricyanide-nitroprusside)	Pink	Pink	Pink	Pink
Sakaguchi[d]	Neg.	Neg.	Neg.	Neg.
Diacetyl-α-naphthol[e]	Blue	Neg.	Neg.	Blue
Ninhydrin	Yellow	Neg.	Neg.	Yellow

[a] Chromatograms were run on precoated silica gel glass plates. The samples were tested at 25 ug per spot.

[b] Saxitoxin and minor toxin were chromatographed in solvent system A.

[c] Major toxins H and L were chromatographed in solvent system E.

[d] At a level of 25 ug per spot both major toxin H and L gave a very faint yellow spot.

[e] Both major toxins H and L gave a very weak purple spot at 25 ug per spot.

each case the active component of minor toxin co-chromatographed with saxitoxin and gave identical color reactions. Impurities in the minor toxin showed up as additional spots (different R_f values) when plates were observed under long wave UV light or treated with the Sakaguchi, diacetyl-α-naphthol, H_2SO_4 or NBD-Cl reagents. The color reactions given by a number of guanidine derivatives are shown in Table 15. Although saxitoxin is known to contain the guanidine residue, its presence in G. tamarensis major toxin has not been established or ruled out. However, evidence presented in the following section suggests that major toxin and saxitoxin are structurally related, and therefore, that the guanidine residue may be present in the major toxin. The essentially negative Sakaguchi test given by the major toxin would rule out a mono-substituted guanidine. The negative Jaffe and Benedict-Behre would tend to rule out a carbonyl function adjacent to one of the guanidinum nitrogen atoms as is present in creatinine which gives a positive reaction with the latter two sprays. Since the Weber and diacetyl-α-naphthol tend to give more positive reactions with the less highly substituted guanidines, the negative test by major toxin in the diacetyl-α- naphthol reaction may indicate an additional mode of substitution present on a guanidine residue not present in saxitoxin since the latter compound shows a positive reaction with this reagent.

Stability and Interconversion of the Toxins

Major toxins H and L were each diluted to a concentration of 2 ug/ml with either 10 mM NaH_2PO_4 or 10 mM $NaHCO_3$. These dilutions, which gave a final pH of about 4.7 and 8.2, respectively, were placed in a water bath at 16^o for 30 hours or 93^o for 30 minutes. After the incubation period, the samples at pH 8.2 were adjusted to pH 4-5 with

TABLE 15. Color Reactions Given by Guanidine Derivatives with Several Spray Reagents

	Jaffe[a]		Diacetyl-α-naphthol[b]		Benedict-Behre[c]		Sakaguchi[d]		Webber[e]	
	5 ug	25 ug	5 ug	25 ug	5 ug	25 ug	5 ug	25 ug	5 ug	25 ug
UNSUBSTITUTED										
Guanidine	-	+	+	+	-	+	-	-	Orange +	+
MONOSUBSTITUTED										
Arginine	-	-	+	+	-	-	+	+	Pink +	+
Methylguanidine	-	+	+	+	-	-	+	+	+	+
Guanidoacetic Acid	-	+	+	+	-	-	+	+	+	+
Streptomycin	-	+	+	+	-	-	+	+	+	+
N-Acyl										
Sulfaguanidine	-	+	+	+	-	+	-	-	Blue +	+
N-O										
Canavanine	-	-	+	+	-	-	-	-	Purple +	+
N-N										
Aminoguanidine	-	+	+	+	-	+	-	-	+	+
DISUBSTITUTED										
Creatine	-	-	+	+	-	+	-	-	+	+
Arginino Succinic Acid	-	-	-	-	-	-	-	-	-	-
TRISUBSTITUTED										
Creatinine	+	+	-	-	+	+	-	-	-	-
Phosphocreatine	-	-	-	-	-	-	-	-	Pink +	+

TABLE 15. (cont'd)

[a] A positive reaction in the Jaffee test is an orange spot.

[b] A positive reaction with the Diacetyl-α-naphthol spray is a pink or orange spot.

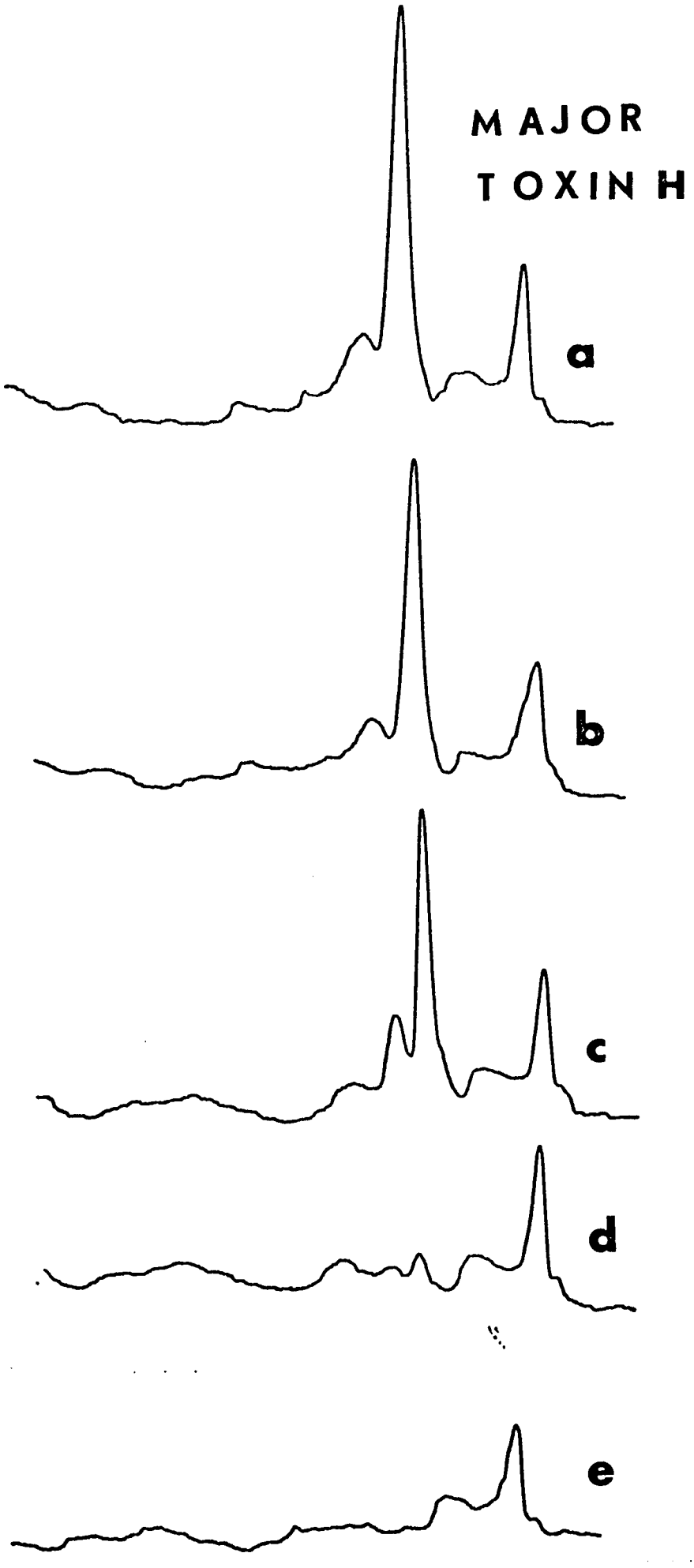
[c] A positive reaction with the Benedict-Behre spray is a purple or orange spot.

[d] A positive reaction with the Sakaguchi spray is a pink spot.

[e] A positive reaction with Weber spray is a spot with variable color.

HCl. One half of each sample was used for the mouse assay, the other half was lyophilized and the residue taken up in sufficient distilled water to give a final concentration of 40 ug/ml. These latter preparations were then evaluated using the TLC-fluorometric assay (Fig. 16 and Table 16). At pH 4.7 major toxin H appeared to be unaffected even after heating at 93°C for 30 minutes; while major toxin L was partially converted to major toxin H at both 16°C and 93°C. At pH 8.2, both major toxins H and L were destroyed by heating, conditions which resulted in the appearance of a small peak corresponding to the position of STX on the TLC plate. Major toxin H appeared to be unchanged after 30 hours at pH 8.2 at 16°C.

Fig. 16. Scans of TLC plates from a pH-temperature stability study of major toxin H and major toxin L under the following conditions: (a) pH 4.7, 16°C for 30 hr; (b) pH 4.7, 93°C for 30 min; (c) pH 8.2, 16°C for 30 hr; (d) pH 8.2, 93°C for 30 min. Scan (e) is a blank. (Refer to Table 16 and the text for further details.)


MAJOR
TOXIN H
a
b
c
d
e

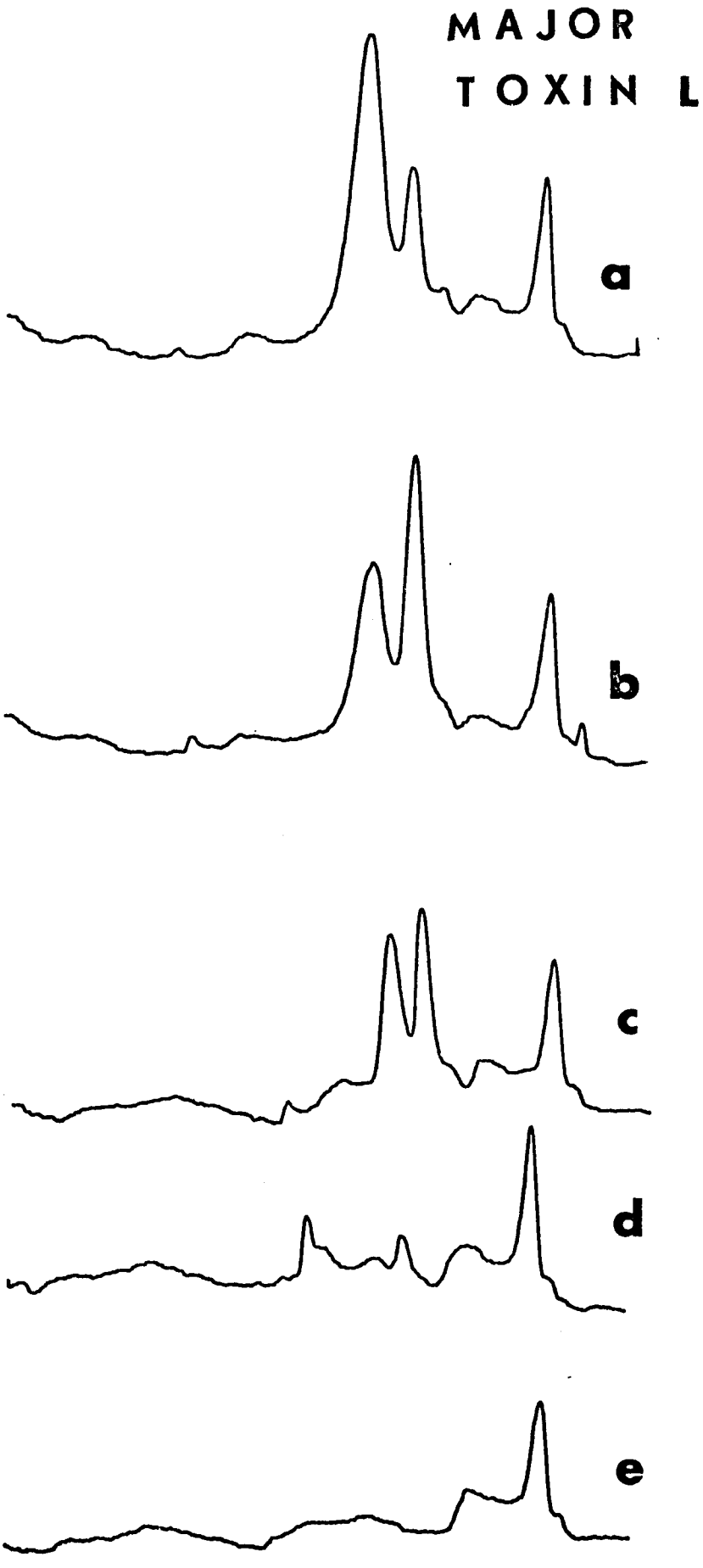

MAJOR
TOXIN L
a
b
c
d
e

TABLE 16. Stability of Major Toxins H and L

Conditions			TLC-Fluorometric Assay[a]				Mouse Test
pH	Temp. (°C)	Time (hr)	Concentration (ug/ml)			Activity[b] (MU/ml)	Activity (MU/ml)
			MA H	MA L	STX		
			Major Toxin H				
4.7	16	30	34	4	0	3.9	3.6
4.7	93	.5	36	4	0	4.0	4.6
8.2	16	30	40	0	0[c]	3.6	4.0
8.2	93	.5	0	0	0[c]	0	0
			Major Toxin L				
4.7	16	30	17	18	0	5.3	7.6
4.7	93	.5	28	15	0	5.6	7.1
8.2	16	30	14	8	9	5.4	7.1
8.2	93	.5	0	0	0[c]	0	0

[a] The *in situ* TLC-fluorometric assay was done on solutions concentrated to give an initial concentration of major toxin H or L of 40 ug/ml (see text).

[b] Calculated by multiplying ug MA H by 1.8, ug MA L by 4.2, and ug STX/ml by 5.5 and dividing the sum by 20 to correspond to the initial concentration of 2 ug per ml used in the mouse assay.

[c] Although a small peak was discernable, its value was negligible (see Fig. 16).

DISCUSSION

Unlike G. catenella and shellfish exposed to G. catenella which appear to contain only saxitoxin, extracts of G. tamarensis and shellfish exposed to G. tamarensis appear to contain a mixture of at least three toxins. Table 17 shows a summary of the results obtained by different researchers during the isolation of poison from shellfish exposed to a "red tide". In agreement with earlier reports on the behavior of G. tamarensis poison from scallops (8), mussels (56) Table 17) and cultures of G. tamarensis (3) the majority of poison in our extracts of Mya arenaria was not firmly bound to sodium Amberlite IRC-50 columns but was eluted with pH 4 acetate buffer along with the majority of inert material. A recent report by Shimizu et al. (70) confirms this observation.

Our results (Tables 2 and 3) show that three active fractions were eluted from the sodium Amberlite IRC-50 column. The first active fraction which was eluted during the sample application and first water rinse contained about 18% of the activity applied. The second active fraction (major toxic fraction), accounting for 35% of the applied activity, was eluted with pH 4 acetate buffer. The third active fraction (minor toxic fraction), eluted with 0.5M acetic acid, accounted for 25% of the applied activity. Shimizu et al. (70) reported the loss of a large portion of the toxicity in the fraction eluted with sodium acetate buffer (major toxic fraction), apparently due to the strong basicity of the eluate. In our work this problem was minimized by washing the sodium Amberlite IRC-50 resin with sufficient deionized distilled water to bring the pH of the eluate down to

TABLE 17. Summary of the Results Obtained During Isolation of the Toxic Substances, Present in Shellfish Exposed to a 'Red Tide', by the Schantz *et al.* (32) Procedure.

	This Thesis[a]		Schantz (1957)	Evans (1970)
Starting Material	E-2	E-3		
whole fresh weight (kg)	10	23	-	5
meat and juice (kg)	3.3[a]	11.0	-[a]	2.1
Celite Filtrate				
solids (g)	21.8	786	2,900	-
total activity ($MUx10^5$)	1.63	4.72	57	.85
specific activity (MU/mg)	7.5	.6	2	-
Amberlite IRC-50 column				
Major Toxic Fraction[b]				
solids (g)	71.1	282	-	-
total activity ($MUx10^4$)	5.2	12.4	-	3.8
specific activity (MU/mg)	.7	.44	-	-[c]
Minor Toxic Fraction				
solids (g)	1.05	9.2	13.5[d]	-
total activity ($MUx10^4$)	2.7	10.6	540	1.5
specific activity (MU/mg)	26.3	11.5	400	-
Amberlite CG-50 column				
Minor Toxin				
solids (mg)	24.8	64.6	5,400	1.8
total activity ($MUx10^4$)	3.2	8.9	480	.28
specific activity (MU/mg)	1,310	1,383	890	1,550

[a] Strained meat

TABLE 17. (cont'd)

[b] This toxic fraction is found only in shellfish exposed to G. tamarensis. It is not present in the Schantz column because this work was performed on G. catanella-exposed shellfish from the West Coast.

[c] The specific activity was not given at this stage. Repeated runs on a Sephadex G-10 column brought the activity to 270 MU/mg. This sample was estimated to be 90% NaCl.

[d] minus ash content

pH 8-9 prior to sample application and by adjusting the acetate buffer eluate to pH 4 with HCl immediately after elution from the column. When material from the first or second toxic fractions was rechromatographed on a smaller sodium Amberlite IRC-50 column, two toxic fractions were obtained: one eluted during the sample application and first water rinse; and a second eluted with pH 4 acetate buffer, corresponding to the major toxic fraction. When material from the third toxic fraction (minor toxic fraction) was rechromatographed on a smaller sodium Amberlite IRC-50 column only one peak of activity was eluted. This peak eluted with 0.5 M acetic acid and corresponded to the minor toxic fraction. These observations suggested that at least two poisons were present in our crude extracts. It is possible that the active material eluted during the sample application and first water rinse was not identical to major toxin. However, the active components in the first and second toxic fractions are most likely identical, the first toxic fraction probably simply arising from saturation of the resin.

The ratio of activity in the minor toxic fraction to the activity in the major toxic fraction varies widely between reports. This variability may be due to several factors including: (1) the source of poison (clams, mussels or cultures of G. tamarensis); (2) the portion of the shellfish used; (3) the length of time between the occurrence of a "red tide" and collection of the shellfish; (4) the time between collection and subsequent work up; and (5) the particular methodology used. We have observed a gradual increase in the activity of the minor toxic fraction relative to the major toxic fraction in three consecutive runs of the sodium Amberlite IRC-50 column over a six month period. The starting material for all three runs was the same batch of clams collected during the 1972 New England

"red tide". A similar observation led Ghazarossian et al. (59) to speculate that G. tamarensis produces a less basic precursor (major toxin) which breaks down into saxitoxin (minor toxin) on aging.

The active component present in the minor toxic fraction behaved similarly to saxitoxin on hydrogen Amberlite CG-50 columns. Following the procedure of Schantz et al. (32) for the isolation of saxitoxin, G. tamarensis minor toxin has been purified to a specific toxicity as high as 2,800 MU/mg (Table 4). This is about one half the toxicity of pure saxitoxin (33) but compares favorably with the activity of saxitoxin at this stage in the isolation procedure. Chromatography on an alumina column employed in the further purification of saxitoxin was not attempted due to the low yields reported for this step (32). TLC studies were used to test the hypothesis that G. tamarensis minor toxin and saxitoxin were identical. In every solvent system employed, G. tamarensis minor toxin co-chromatographed with saxitoxin. The solvent systems used included the four shown in Fig. 3, solvent system E (Fig. 10, Table 8) and numerous other solvent systems tested for suitability in the TLC-fluorometric assay. The identical color reactions given by saxitoxin and G. tamarensis minor toxin (Table 14) further support this hypothesis. Several of these spray reagents are very specific for certain types of substituted guanidines (Table 15). The H_2O_2 fluorescent derivatives of saxitoxin and G. tamarensis minor toxin co-chromatographed in solvent systems B and E (Fig. 12) and had identical excitation and fluorescence maxima (Table 8) within the limits of experimental variation. These results provide strong but no compelling evidence that the poison eluted from our sodium Amberlite IRC-50 columns with 0.5 M acetic acid (G. tamarensis

minor toxin) is identical to saxitoxin. Ghazarossian et al. (52) drew a similar conclusion from a study of the poison in ten year old extracts of the hepatopancreas of scallops exposed to a "red tide" of G. tamarensis. Physiological studies by Evans (56, 7) confirm this conclusion since he has reported finding no differences in the action of saxitoxin or G. tamarensis minor toxin in a variety of nerve and muscle preparations.

The ethanol extraction and Bio-Gel columns described (71) provided the first successful procedure for the separation of the active components present in the major toxic fraction from the bulk of inert material. Our TLC studies of G. tamarensis major and minor toxins (71) provided the first decisive evidence that shellfish exposed to G. tamarensis contain a mixture of at least two poisons. The physiological studies of Evans (56, 7) revealed only subtle differences between saxitoxin (or minor toxin) and major toxin in their effects on test physiological preparations. These small differences may not be significant, however, since the preparation of major toxin used was at best only 10% pure. The differential absorption of poison to the sodium Amberlite column might possibly be explained by interaction of the poison with other molecules, such as binding with proteins. However, TLC of G. tamarensis major and minor toxins (Figs. 4 and 10, Table 8) and the different color reactions given by these toxins (Table 14) provide compelling evidence that they are chemically different. Although the excitation and fluorescence maxima (Table 8) of the H_2O_2 derivatives of major and minor toxin are similar; the R_f values of these derivatives are markedly different (Fig. 12, Table 8).

These TLC studies showed further that major toxin prepared

by the procedure described contains a mixture of two toxic components. These two components, called major toxin H and major toxin L, appear to be chemically very similar. The fact that these two compounds were carried through several chromatographic steps without separation is a strong indication of their similarity. Major toxin H and L gave similar color reactions with all the spray reagents tested and the H_2O_2 fluorescent derivatives of both toxins had identical excitation and fluorescence maxima and had identical R_f values in solvent system E (Fig. 12, Table 8). Furthermore, major toxin L is converted to major toxin H or a substance co-chromatographing with major toxin H under a variety of mild conditions (Fig. 16). There is no indication that this conversion is reversible, although this possibility cannot be ruled out. There is also some evidence that suggests that both major toxins may break down to give STX (Fig. 16), further indicating their structural similarity.

One of the main goals of our research has been to develop a rapid, simple method for the detection and quantitation of PSP in crude clam extracts and samples generated during the isolation of these compounds. Using the mouse test to evaluate the hundreds of fractions collected during just one column chromatographic run is an expensive and time consuming process. Also, the mouse test is relatively non-specific and gives no indication which of the G. tamarensis toxins is present. The Jaffe test (19) was tried in an attempt to evaluate fractions from the Bio-Gel column, but no correlation was observed between toxicity as determined by the Jaffe and mouse tests.

Since we were able to get reasonably good separation of the G. tamarensis poisons on TLC, several spray reagents were screened for one specific and sensitive enough to detect the poisons in unconcentrated

column eluate. None of the TLC spray reagents previously described for saxitoxin were sufficiently sensitive. Attempts to make fluorescent derivatives of the poisons by reaction with NBD chloride (72) were unsuccessful. Reaction of the poisons on TLC plates with NBD chloride according to the method of Reisch et al. (64) for sulphonamides produced colored spots but this technique was not sufficiently sensitive or specific. Dansyl derivatives of the poisons were also produced (73) but again this reaction was not specific for the poisons.

Wong et al. (54) reported the structure of a derivative of saxitoxin produced by oxidation of saxitoxin with 0.8% hydrogen peroxide in solution at 25°C. There was some indication that the derivative, a pyrimido (2, 1-b)-purine (Fig. 1, structure 5) was fluorescent. We succeeded in producing fluorescent derivatives of saxitoxin and the G. tamarensis poisons on TLC plates by spraying the plates with 1% hydrogen peroxide. The reaction proceeded very slowly at room temperature, but it was found that heating the plates immediately after spraying greatly accelerated the production of the fluorescent spots. The H_2O_2 spray proved to be a very sensitive and specific TLC spray reagent for the detection of PSP. Using this technique, as little as 0.1 n moles of saxitoxin, or about 0.2 MU are easily visible on a TLC plate under long wave UV light. Active fractions from a column can be located simply by spotting 4 ul aliquots of each fraction to a TLC plate, spraying the plate with 1% hydrogen peroxide, heating the plate and observing the plate under UV light. Fractions which show activity in the mouse test give light blue fluorescent spots. Inactive fractions show no fluorescence. Since no development of the plates is necessary to determine which fractions are fluorescent hundreds of fractions can be screened on one plate in a fraction of the time re-

quired to evaluate the individual fractions with the mouse test.

Attempts to quantitate the individual paralytic shellfish poisons were readily successful once a solvent system capable of giving good separation of major toxin H, and major toxin L and saxitoxin was found. Using solvent system E and the standard procedure described above, as little as 40 ng of the poisons can be quantitated. The sensitivity of the assay can be extended by increasing the sensitivity of the fluorometer (to 30x) or by replacing the 47 B secondary filter in the fluorometer with a 2A filter; however, both of these changes give a more irregular background trace. Possibly by scanning perpendicular to the direction of the spot migration a reasonable base line can be established and the sensitivity of the in situ TLC-fluorometric assay might be increased by a factor of about three.

The chemical assay for saxitoxin recently described by Bates and Rapoport (22) also involves reaction of the poison with H_2O_2. However, they carried out the reaction in solution without heating. Whether or not the same fluorescent derivatives are involved is still open to question, although the excitation and fluorescence maxima reported are similar to the values obtained here (Table 8). Their assay was successfully applied to a number of samples of shellfish collected on the Pacific coast which presumably contained only saxitoxin, acquired from feeding on G. catenella. The applicability and suitability of this assay to shellfish exposed to G. tamarensis on the Atlantic coast, which apparently contain at least two toxins in addition to saxitoxin, is uncertain. The Bio-Rex 70, a weak cation exchange resin, used in the preliminary clean-up of the sample by Bates and Rapoport (22) would probably not bind the majority of the G. tamarensis poison. Furthermore, since these shellfish contain a mixture of poisons, quan-

titation by the method described without prior separation of the individual poisons would be difficult. The lengthy procedure required for the preliminary clean up of the samples makes it doubtful whether this procedure will gain wide use.

The in situ TLC-fluorometric assay we described has great potential as a research tool for the study of PSP. Development of this assay greatly facilitated the isolation of the G. tamarensis poisons. Without this technique, it probably would not have been possible to separate major toxin H from major toxin L. Since the in situ TLC-fluorometric assay allows quantitation of each of the G. tamarensis poisons individually, it can be used to answer some important questions concerning relationships between the poisons. For example: Are all three of the poisons produced by G. tamarensis?; Is one of the poisons a precursor of the others?; Is there actually some conversion upon aging of a weakly basic or neutral substance to saxitoxin? In addition to its great utility as a research tool, the in situ TLC-fluorometric assay may prove useful in the routine screening of shellfish if a simple method for preliminary clean up of the extracts can be developed.

The 1972 and 1974 outbreaks of PSP can no longer be regarded as isolated incidents. Residual concentrations of these poisons persist for long periods in the marine environment and given the proper set of conditions, the causative organism, G. tamarensis could again reach toxic concentrations. In fact, the possibility would seem to be even greater due to the large seed population left behind by previous "red tides". Along with tetrodotoxin and saxitoxin, the G. tamarensis poisons are among the most toxic substances known. It has been estimated that consumption of fewer than six clams during the 1972 outbreak would have been sufficient to cause death. The availability

of purified G. tamarensis poisons should aid in our understanding of PSP. Knowledge of the properties of these poisons may provide information for the preparation of marginally toxic shellfish for consumption.

The poisons produced by G. tamarensis are of considerable biomedical interest. In addition to their potential as model compounds for new drugs, they may serve as powerful tools for use in research into membrane physiology and the central and peripheral nervous systems. Information about the structure of G. tamarensis poisons will aid in determining structure-function relationships in the group of marine biotoxins including saxitoxin and tetrodotoxin. Tetrodotoxin, saxitoxin and the G. tamarensis poisons affect the process whereby the nerve membrane undergoes an increased permeability to sodium ions upon stimulation, thereby blocking nervous conduction (74, 75). When the structures of the G. tamarensis poisons are completely identified, common features in the structure of these four toxins may give information on the structure of the receptor and sodium channel. It may also provide information about their metabolism (76) and function within the producing organisms, both of which are presently unknown.

SUMMARY

Three toxins have been purified from clams (Mya arenaria) that had become toxic during the 1972 G. tamarensis red tide in the central New England coastal waters. All three are potent neurotoxins and produce effects identical to saxitoxin when injected into mice or killifish (Fundulus heteroclitus). The majority of the poison present in the extracts was apparently less basic than saxitoxin since it was not firmly bound to the weak cation exchange resins used in the initial step of the purification procedure.

The active component present in the firmly bound fraction, minor toxin has been purified to a potency of 2,800 MU per mg and identified as saxitoxin. This identification was based on the following data: 1) behavior on weak cation exchange resin columns; 2) TLC of minor toxin and authentic saxitoxin in numerous solvent systems; 3) color reactions given by minor toxin and authentic saxitoxin with several specific spray reagents; 4) TLC of the hydrogen peroxide-fluorescent derivatives of minor toxin and authentic saxitoxin; 5) the spectral properties of minor toxin and authentic saxitoxin and their fluorescent derivatives and 6) the effects of minor toxin and authentic saxitoxin on test organisms.

Two additional toxic components, major toxin H and major toxin L, have been isolated from the weakly bound fraction (major toxic fraction). The active components were separated from the bulk of inert material by a series of extractions into cold ethanol followed by passage through a Bio-Gel P-2 column.

A new in situ TLC-fluorometric assay has been developed that

provides a means of distinguishing between major toxin H, major toxin L and saxitoxin (minor toxin), quantitating each toxin individually, and estimating their combined potency. This assay involves TLC of the samples along with the appropriate standards on silica gel plates, followed by reaction of the poisons on the plate with 1% hydrogen peroxide at 100°C. The plates are then scanned with a fluorometer. A linear relationship between peak height and the amount of poison spotted has been observed for all three paralytic shellfish poisons over the range of 40 to 400 ng poison per spot. As little as 40 ng (0.2 MU, 0.1 nm) of saxitoxin can be quantitated. A simplified procedure involving application of 4 ul of sample in rows 1 cm apart to a portion of a silica gel plate and reaction with hydrogen peroxide without development in any solvent system has been used to locate active fractions eluted from the Bio-Gel columns.

Separation of major toxin H from major toxin L by repeated passes through a Bio-Gel column was greatly facilitated by the development of the TLC-fluorometric assay. Major toxin H was purified to a maximum potency of 1,800 MU per mg; while major toxin L was purified to a potency of 4,200 MU per mg. Preparations of both toxins were shown to be chromatographically pure by TLC in several solvent systems.

The data suggests that major toxin H and major toxin L are chemically similar and that they are also probably related to saxitoxin. The similarity of major toxins H and L has been demonstrated by TLC of their hydrogen peroxide-fluorescent derivatives, the spectral properties of these derivatives and the pH-temperature stability studies where major toxin L appeared to undergo a partial conversion to major toxin H. The similarity between both major toxins H and L and saxi-

toxin has been demonstrated by the apparent conversions of the major toxins to saxitoxin, their similar physiological action and the similar fluorescence properties of their derivatives.

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